

SEPARATION AND CHARACTERIZATION OF RECONSTITUTED SKIM MILK  
POWDER TREATED WITH MINERAL CHELATORS

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by  
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## ABSTRACT

### Separation and Characterization of Reconstituted Skim Milk Powder Treated with Mineral Chelators

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The proteins found in milk are largely important in the functionality of many dairy products and dairy processes. The casein micelle system in milk is a complex and highly studied system. The micelle is thought to be a sponge like structure containing four caseins,  $\alpha_{s1}$ ,  $\alpha_{s2}$ ,  $\beta$ , and  $\kappa$  casein, and bound together with colloidal calcium phosphate. When a chelating agent such as a citrate, phosphate, or polyphosphate are added to milk systems, the CCP is bound to the chelator and removed from the micelle. It has been shown through past research that the use of calcium chelating agents disrupts the calcium phosphate equilibrium and allows for the dissociation of the casein micelle and release of the individual caseins. Once the caseins are disrupted from micellar form and in solution, it may be possible to separate out different casein streams for functional usage in dairy products using common separation techniques.

This thesis project seeks to evaluate the feasibility of separating milk treated with calcium chelators using various separation techniques to evaluate the individual casein fractions of this disrupted system. Four separation methods (ultracentrifugation, membrane filtration, heat coagulation, and coagulation based on pH) were employed to separate out the caseins based on selected properties, specifically density, molecular weight, and solubility. In ultracentrifugation, three speeds were tested, the heat coagulation study tested two temperatures, and pH based coagulation tested four different pHs to determine their impact on overall protein levels and individual casein yields. Skim

milk powder was reconstituted and chelator was added at 1, 50, or 100 mEq/L treatment level. These samples were then separated using aforementioned techniques, and the supernatant or permeate was analyzed for total protein content, individual casein composition, turbidity, and mean particle size.

Analysis of centrifugal separation studies shows the interaction between chelator type, chelator level, and centrifugation speed had a significant impact on the amount of protein released from the casein micelle ( $p < 0.001$ ). Samples treated with 50 mEq of sodium hexametaphosphate and centrifuged at 60,000xg released significantly more protein into the supernatant than other samples. The type of chelator was not found to be statistically significant in impacting the proportion of  $\alpha$  casein or  $\beta$  casein in the samples, but the interaction of chelator level and centrifugal force was found to have an impact. Larger proportions of  $\alpha$  casein and lower proportions of  $\beta$  casein were seen in samples treated with low levels of chelator (1 mEq) and low levels of centrifugal force (30,000xg). Although the type of chelator used was not seen to cause significant difference in the supernatant of samples, it is important to note that when comparing the chelated samples to the control samples, drastic differences were seen in the turbidity, protein content, and casein composition of samples, suggesting that while the type of chelator does not significantly impact these things, the use of a chelator does.

Coagulation trials based on pH were also shown to have a significant interaction between chelator type, chelator level, and sample pH effecting the protein levels and casein composition ( $p < 0.01$ ). Significantly more protein was found in samples with higher pH, suggesting that as the pH decreases past the isoionic point of each individual casein, it precipitates out of solution. The proportion of  $\beta$  casein and  $\kappa$  casein varied

inversely as the pH was adjusted, with high percentages of  $\beta$  casein ( $40.76\% \pm 2.66$ ) and low percentages of  $\kappa$  casein ( $10.14\% \pm 1.52$ ) found in the samples with a pH of 5.37. An interesting change in particle sample size was seen in samples treated with 100 mEq SHMP at pH 5.37. These samples had an average particle size 200  $\mu\text{m}$  larger than other samples.

Membrane filtration showed low protein yields in permeate, however trisodium citrate 100 mEq was still shown to have significantly higher permeate % protein levels ( $p < 0.001$ ), with a mean protein reading of 0.054% to 0.061%. Casein content was not able to be analyzed, as there was minimal casein present in the permeate. Further work in this area should be continued using a larger membrane pore size.

The use of heat based coagulation as an individual casein separation technique for chelated samples is not recommended, as the casein micelle system itself is extremely heat stable, and the use of calcium chelators only increases the heat stability further. Because of the increased heat stability, no coagulum was formed in samples upon heating, and therefore, no separation and analysis could be done.

Improving our knowledge of pretreatment of milk prior to separation and the effectiveness of different separation methods on chelated milk products may result in information leading to the ability to separate out milk fractions that provide unique or improved properties for product applications.

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## TABLE OF CONTENTS

	Page
LIST OF TABLES .....	xiv
LIST OF FIGURES .....	xvii
CHAPTER	
1. INTRODUCTION .....	1
2. LITERATURE REVIEW .....	3
2.1 Introduction .....	3
2.2 Casein Micelle .....	3
2.2.1 Introduction to Casein Micelles.....	3
2.2.2 Composition of Casein Micelles.....	4
2.2.3 Suggested Models.....	5
2.2.4 $\alpha$ caseins.....	9
2.2.5 $\beta$ casein .....	9
2.2.6 $\kappa$ casein .....	10
2.2.7 Colloidal Calcium Phosphate .....	11
2.3 Chelators.....	12
2.3.1 Citrates.....	14
2.3.2 Phosphates .....	15
2.3.3 Ethylenediaminetetraacetic Acid.....	16

2.4 Separation.....	17
2.4.1 Size Based Separation .....	18
2.4.1.1 Membrane Filtration .....	18
2.4.1.2 Centrifugation .....	20
2.4.1.3 Gel Permeation Chromatography .....	21
2.4.2 Solubility .....	24
2.4.2.1 Heat Coagulation .....	24
2.4.2.2 Acidification .....	25
2.4.3 Ion Exchange Chromatography .....	27
2.5 Summary .....	29
3. JUSTIFICATION AND HYPOTHESIS .....	31
4. MATERIALS AND METHODS.....	33
4.1 Experimental Design .....	33
4.1.1 Treatment Level Determination.....	34
4.1.2 Preliminary Power Study.....	35
4.1.3 Ultracentrifugation Study .....	38
4.1.4 Solubility with Temperature .....	39
4.1.5 Membrane Filtration Study.....	40
4.1.6 Acidification Study.....	40
4.1.7 Data Analysis.....	41

4.2 Rehydration of Skim Milk Powder .....	42
4.3 Chelation .....	42
4.4 Separation Methods.....	43
4.4.1 Ultracentrifugation.....	43
4.4.2 Temperature.....	45
4.4.3 Membrane Filtration .....	45
4.4.4 Acidification .....	46
4.5 Analytical Methods .....	47
4.5.1 Total Nitrogen and Protein .....	47
4.5.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)..	48
4.5.3 Turbidity .....	49
4.5.4 Particle Size Analysis .....	49
5. RESULTS AND DISCUSSION.....	50
5.1 Unseparated Sample Analysis .....	50
5.1.1 Turbidity .....	50
5.1.2 Particle Size Analysis .....	52
5.1.3 pH .....	53
5.2 Centrifugation.....	54
5.2.1 Turbidity .....	54
5.2.2 Protein Content.....	57

5.2.3 Alpha Casein.....	60
5.2.4 Beta Casein .....	62
5.2.5 Kappa Casein .....	63
5.2.6 Particle Size Analysis .....	65
5.3 Temperature .....	65
5.4 Membrane Filtration.....	65
5.4.1 Turbidity .....	65
5.4.2 Protein Content .....	67
5.4.3 Casein Content.....	69
5.4.4 Particle Size Analysis .....	70
5.5 Acidification.....	71
5.5.1 Turbidity .....	71
5.5.2 Protein Content .....	72
5.5.3 Alpha Casein.....	74
5.5.4 Beta Casein .....	76
5.5.5 Kappa Casein .....	77
5.5.6 Particle Size Analysis .....	79
6. CONCLUSIONS AND FURTHER RESEARCH.....	82
REFERENCES .....	85
APPENDICES	

Appendix A- Unseparated Sample Raw Data.....	96
Appendix B- Centrifugation Study Raw Data .....	97
Appendix C- Membrane Filtration Study Raw Data .....	100
Appendix D- Acidification Study Raw Data .....	101
Appendix E- Unseparated Sample Minitab Output .....	104
Appendix F- Centrifugation Study Minitab Outputs .....	110
Appendix G- Membrane Filtration Study Minitab Outputs.....	120
Appendix H- Acidification Study Minitab Outputs.....	123

## LIST OF TABLES

Table	Page
2.1. Average physical properties of casein micelle (Adapted from Fox and Brodkorb, 2008) .....	5
2.2. Isoionic pH of casein protein variants adapted from Swaisgood (1993) and Fox (2003) with reference variant of each casein bolded .....	27
4.1. Definition of terms for experimental design for preliminary power study. SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate, EDTA = Ethylenediaminetetraacetic Acid .....	36
4.2. Treatment definitions for 2x3x3 full factorial ultracentrifugation study. SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate .....	38
4.3. Treatment definitions for 2x3x2 full factorial solubility with temperature study. SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate .....	39
4.4. Treatment definitions for 2x3 full factorial membrane filtration study. SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate .....	40
4.5. Treatment definitions for 2x3x4 full factorial acidification study. SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate .....	41
4.6. Chelator Concentrations in mmol/L, mEq/L, and (g chelator/g SMP) .....	43
5.1. Mean ( $\pm$ pooled SE) protein levels (%) for centrifuged supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ). Control included for observation only, not as part of balanced design with factorial treatment structure. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate .....	59

5.2. $\kappa$ casein proportion ( $\% \pm$ pooled SE) of centrifuged supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ). Control included for observation only, not as part of balanced design with factorial treatment structure. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate .....	64
5.3. Mean turbidity at 700nm ( $\pm$ pooled SE) for membrane filtered permeate. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ). Control included for observation only, not as part of balanced design with factorial treatment structure. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate .....	67
5.4. Mean protein levels ( $\% \pm$ pooled SE) for membrane filtered permeate. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ). Control included for observation only, not as part of balanced design with factorial treatment structure. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate .....	68
5.5. Turbidity ( $\pm$ pooled SE) at 700nm for pH adjusted supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ). Control included for observation only, not as part of balanced design with factorial treatment structure. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate .....	72

5.6. Mean protein levels ( $\%$ $\pm$ pooled SE) for pH adjusted supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ). Control included for observation only, not as part of balanced design with factorial treatment structure. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate .....	74
5.7. Mean particle size ( $\mu\text{m}$ $\pm$ pooled SE) for pH adjusted supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ). Control included for observation only, not as part of balanced design with factorial treatment structure. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate .....	81



## LIST OF FIGURES

Figure	Page
2.1. Isosurface of 35nm slab of casein micelle, showing lattice type structure of micelle including channels and voids, pictured by Trejo et al. using cryoelectron microscopy (Trejo et al., 2011).....	7
2.2. Casein micelle internal structure model (De Kruif and Holt, 2003).....	8
2.3. Membrane filtration classifications (Brans et al., 2004).....	19
2.4. Example of a) dead end filtration and b) cross flow filtration (Saxena et al., 2009) .....	20
2.5. Example of gel permeation chromatography (Lehninger et al., 2008) .....	23
2.6. Example of cation exchange chromatography showing a positively charged resin binding negatively charged molecules (Lehninger et al., 2008) .....	28
4.1. Equations used by Minitab 17.0 statistical software for analysis of statistical power using “Power and Sample Size for General Full Factorial Design” command.....	37
4.2. Power curve showing that in four data replicates, 92% chance of correctly detecting difference in % $\kappa$ casein in supernatant of samples .....	37
4.3. Schematic of the placement of $r_{\min}$ , $r_{\text{av}}$ and $r_{\max}$ on a Beckman-Coulter rotor. The $r_{\max}$ was used for all calculations as the pellet forms on the bottom on the centrifuge tube (Beckman-Coulter, 2000) .....	44
4.4. Schematic of Amicon stirred cell membrane filtration unit (EMD Millipore, 2015) .....	46

5.1. Means ( $\pm$ SE) showing the turbidity of the stock (unseparated) chelated samples. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate .....	51
5.2. Means ( $\pm$ SE) showing the mean particle size ( $\mu$ m) of the stock (unseparated) chelated samples. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate .....	53
5.3. Means ( $\pm$ SE) showing the pH of the stock (unseparated) chelated samples. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate .....	54
5.4. Means ( $\pm$ SE) showing the turbidity for the statistically significant effect of chelator level on the centrifuged supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ) .....	56
5.5. Means ( $\pm$ SE) showing the turbidity for the statistically significant effect of centrifugal force on the centrifuged supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ) .....	56
5.6. Means ( $\pm$ SE) showing the proportion of $\alpha$ casein for the statistically significant interaction of chelator level and centrifugal force on the centrifuged supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ).....	61

- 5.7. Means ( $\pm$ SE) showing the proportion of  $\beta$  casein for the statistically significant interaction of chelator level and centrifugal force on the centrifuged supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ) .....63
- 5.8. Denatured and reduced permeate samples run on a 12% SDS-PAGE gel and visualized through Coomassie staining Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate *Lane 1*: Control *Lane 2*: TSC 100 mEq *Lane 3*: SHMP 100mEq *Lane 4*: SHMP 1 mEq *Lane 5*: TSC 50 mEq *Lane 6*: SHMP 50 mEq *Lane 7*: Unfiltered milk *Lane 8*: TSC 1 mEq.....69
- 5.9. Denatured and reduced retentate samples run on a 12% SDS-PAGE gel and visualized through Coomassie staining Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate *Lane 1*: TSC 1 mEq *Lane 2*: TSC 100 mEq *Lane 3*: Unfiltered milk *Lane 4*: SHMP 50 mEq *Lane 5*: SHMP 1 mEq *Lane 6*: Control *Lane 7*: TSC 50 mEq *Lane 8*: SHMP 100 mEq .....70
- 5.10. Denatured and reduced supernatant samples run on a 12% SDS-PAGE gel and visualized through Coomassie staining. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate *Lane 1*: TSC 1 mEq, pH 5.14 *Lane 2*: TSC 100 mEq, pH 5.14 *Lane 3*: TSC 100mEq 5.37 *Lane 4*: SHMP 100Meq, pH 5.61 *Lane 5*: TSC 1 mEq pH 4.94 *Lane 6*: SHMP 1 mEq pH 4.94 *Lane 7*: Control, pH 4.94 *Lane 8*: TSC 1 mEq pH 5.37 *Lane 9*: SHMP 50 mEq pH 4.94 *Lane 10*: TSC 50 mEq pH 5.14 .....75

5.11. Means ( $\pm$ SE) for the statistically significant effect of adjusted pH on mean proportion of $\beta$ casein in sample supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ) .....	77
5.12. Means ( $\pm$ SE) showing the mean proportion of $\kappa$ casein in sample supernatant for the statistically significant effect of chelator level on the pH adjusted sample supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ) .....	78
5.13. Means ( $\pm$ SE) showing the mean proportion of $\kappa$ casein in sample supernatant for the statistically significant effect of pH on the pH adjusted sample supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ) .....	79

## 1. INTRODUCTION

Caseins are the most abundant proteins in milk, composing approximately 80% of the total protein content. In their native state in milk, the caseins are in a micellar structure, bound together with colloidal calcium phosphate. The caseins are bound together in a set proportion of approximately 4:1:4:1 of  $\alpha_{s1}$ ,  $\alpha_{s2}$ ,  $\beta$ , and  $\kappa$  casein, respectively. There is interest in manipulating the individual properties and advantages of the caseins in large scale industrial applications. For example,  $\beta$  casein has been suggested to increase cheese curd firmness and yields if added in surplus to cheese make milk (Huppertz et al., 2006),  $\alpha_{s2}$  casein has been found to have antioxidant properties (Kitts, 2005) and increased amounts of  $\kappa$  casein have been linked to a more heat stable milk product (Jimenez-Flores and Richardson, 1988). High protein products are an increasing consumer trend and the manipulation of casein proportions in fractions promotes a possibility to increase the versatility and functions of dairy products. However, when bound together in the micelle, there is no access to the individual caseins. Therefore, the separation of caseins from a micelle can be most effectively done when the micelle is disrupted first.

If the calcium phosphate system is disrupted, this may make it possible to explore the use of current separation techniques to fractionate  $\alpha_{s1}$ ,  $\alpha_{s2}$ ,  $\beta$ , and  $\kappa$  caseins into separate streams. Previous research has shown that the casein micelle system can be disrupted by use of select mineral chelating agents. This study seeks to determine the feasibility of separating milk caseins from milk systems chelated with phosphate and citrate calcium chelating agents (sodium hexametaphosphate and sodium citrate, respectively). These chelators pull the calcium out of the micelle and disrupt the colloidal

calcium phosphate holding the micelle together, effectively releasing the individual caseins into the milk serum.

The purpose of this thesis is to contribute to the understanding of the impact of calcium chelators on casein micelles. Furthermore, this thesis seeks to explore the effectiveness of separation techniques based on selected properties to separate out unique fractions with varying proportions of individual caseins from a pretreated (chelated) milk product.

## 2. LITERATURE REVIEW

### 2.1 Introduction

The purpose of this literature review is to provide a foundation of the previous research pertinent to this thesis as related to the casein micelle and the work of mineral chelators in the dairy industry. First, the casein micelle composition, characteristics, and structure will be addressed. Special interest will be paid to the individual components of the micelle including the different casein and mineral components. Next, because of their wide use in dairy food systems, the general characteristics, functions and use of chelators will be discussed. Particular attention to citrate and phosphate chelating agents will then be described. Finally, this review will discuss separation methods commonly used in laboratory and industrial separations of dairy and protein systems and the governing principles of these systems. This literature review closes with a summary of chelation and casein separation and its industrial value.

### 2.2 Casein Micelle

#### 2.2.1 Introduction to Casein Micelles

Caseins compose approximately 75-80% of the proteins in milk, and are among the most largely studied of food protein systems (Fox and Brodtkorb, 2008). Originally isoelectric casein was thought to be one homogeneous protein. However, it was demonstrated by in the early 20<sup>th</sup> century that casein was composed of several different fractions. It has since been determined that the overall casein, now referred to as casein micelle, is composed of four different types of casein –  $\alpha_{s1}$ ,  $\alpha_{s2}$ ,  $\beta$ , and  $\kappa$  casein, as well as colloidal calcium phosphate. These caseins are found in the micelle in a ratio of approximately 4:1:4:1, respectively (Fox, 2003). Extensive research into the casein

micelle has been completed and there is much already known. Understanding of the casein micelle is vital to further understanding many dairy processes, including the coagulation of cheese and yogurt products, and the stability of concentrated milk products to heat or ethanol (Fox and Brodtkorb, 2008).

### 2.2.2 Composition of Casein Micelles

The casein micelle is assembled within the Golgi apparatus of the cow's mammary gland as a way to bundle extra calcium and phosphate for delivery from cow to calf in order to support the calf nutritionally (Lucey, 2002). The assembly brings together the four casein types along with calcium, phosphate, magnesium, and citrate to form the micelle (Horne, 2006). The average casein micelle has a molecular mass of approximately  $1.3 \times 10^9$  Daltons and volumosity of  $44 \text{ cm}^3/\text{g}$  (Fox, 2003; Choi et al., 2011; Brans et al., 2004). The size of the overall casein micelle is most widely determined by the amount of  $\kappa$ -casein present in the micelle, with overall micelle sizes ranging from 50nm to 500nm diameter, and average sizes being around 120-200nm (Glantz et al., 2010; McMahon and Oommen, 2008). The average physical properties of the casein micelle are summarized in Table 2.1.

What is commonly referred to as the casein micelle does not fit the standard definition of a micelle, but is instead a highly hydrated, sponge-like, large spherical aggregate with individual casein molecules being held together by hydrophobic bonds and salt bridges (Walstra, 1990; Choi et al., 2011; McMahon and Oommen, 2008). The colloidal calcium phosphate within the micelle acts as a type of cement helping to cross link the caseins (Choi et al., 2011).



Table 2.1. Average physical properties of casein micelle (Adapted from Fox and Brodkorb, 2008)

Characteristic	Value
Diameter	120nm (range: 50-500 nm)
Surface area	$8 \times 10^{-10} \text{ cm}^2$
Volume	$2.1 \times 10^{-15} \text{ cm}^3$
Density (hydrated)	$1.0632 \text{ g/cm}^3$
Mass	$2.2 \times 10^{-15} \text{ g}$
Water content	63%
Hydration	3.7g H <sub>2</sub> O/g protein
Voluminosity	$44 \text{ cm}^3/\text{g}$
Molecular mass (hydrated)	$1.3 \times 10^9 \text{ Da}$
Molecular mass (dehydrated)	$5 \times 10^8 \text{ Da}$
# of peptide chains	$5 \times 10^3$
# of particles/mL milk	$10^{14} - 10^{16}$
Surface of micelles/mL milk	$5 \times 10^4 \text{ cm}^3$
Mean free distance	240 nm

### 2.2.3 Suggested Models

Although casein micelles have been studied for well over 50 years, a set structure has not been determined (Dalglish et al., 2004). The components of the micelle have been identified, as well as possible roles of the components, but the exact structure of the micelle is still heavily debated. Three different models have been proposed. These

models can be described as the submicelle model, the coat core model, and the internally structured model (Guo et al., 2003; Lucey, 2002; Fox, 2003; Schmidt, 1982; Walstra, 1990; Payens, 1966; Horne, 1998; Holt, 1998).

The submicelle model proposed by Schmidt (1982) and Walstra (1990) suggests that within the micelle, there are smaller units that are either  $\kappa$  casein rich or  $\kappa$  casein poor. The  $\kappa$  casein rich units congregate on the exterior of the micelle, while the  $\kappa$  casein poor units are found in the interior micelle.  $\kappa$  casein in the outer subunits creates a “hairy” layer around the entire micelle. These units are linked together by micellar calcium phosphate and have a “raspberry” like appearance because of the small circular subunits connected together. This is the most widely supported model of the three proposed model types, though different variations on this model are proposed by many (Fox, 2003).

The coat core model rejects the idea of subunits, instead placing certain types of casein within the micelle, surrounded by a coat of a different casein. Some suggest that  $\kappa$  casein forms the core and is surrounded by  $\alpha$  and  $\beta$  caseins (Parry and Carroll, 1969), while others suggest that  $\alpha$  and  $\beta$  caseins form an internal network and are surrounded by  $\kappa$  casein and colloidal calcium phosphate (Payens, 1966). These models were some of the first models to be proposed, and as research and technology has improved, have lost traction. Parry and Carroll’s  $\kappa$  casein core and  $\alpha$  and  $\beta$  caseins has been rejected by most as it has been argued that  $\kappa$  casein is the most hydrophilic of the caseins and  $\alpha$  casein is the most hydrophobic, so the caseins would not aggregate in such a way to put the hydrophobic  $\alpha$  caseins in the coat, exposed to the milk serum, while protecting the hydrophilic  $\kappa$  casein.

The third model is the internally structured micelle. As electron microscopy technologies have improved, this model has gained more interest. It is suggested by McMahon and Oommen (2008) that the casein micelle is a web like structure of individual caseins cross linking together and forming a rigid “lattice” structure or matrix. Horne (1998, 2006), Holt (1992), and Visser (1992) have proposed variations of this dual binding system in which the caseins are linked together both by hydrophobic bonds and colloidal calcium phosphate salt bridging. Cryo-electron microscopy work done by Trejo et al. (2011) supports the internally structured micelle model, as well as providing detailed 3D visualizations showing the presence of nanoclusters and water filled cavities within the micelle (Figure 2.1).

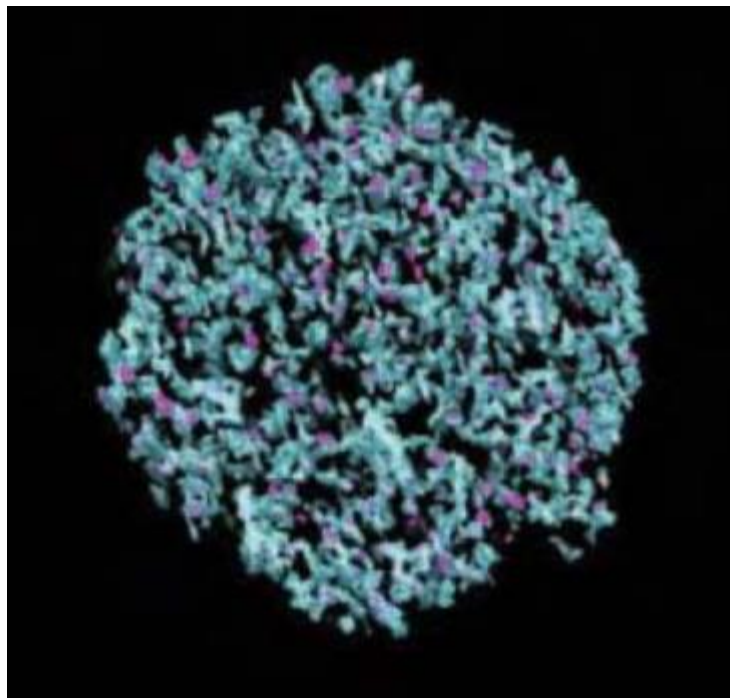


Figure 2.1. Isosurface of 35nm slab of casein micelle, showing lattice type structure of micelle including channels and voids, pictured by Trejo et al. using cryoelectron microscopy (Trejo et al., 2011)

All of these models agree upon the components of the micelle being the four caseins, comprising 94% of the micelle, and the mineral constituents of calcium, phosphate, magnesium and citrate creating the remaining 6% of the structure (Guo et al., 2003). It is also generally agreed, with the exception of Parry and Carroll's work, that  $\kappa$ -casein must be found on or around the surface of the micelle, and that the micelle properties can be changed by altering the pH or temperature of the milk system. An example of this is the tendency of  $\beta$  casein to precipitate out of the system at lower temperatures (Fox, 2003). For the purpose of this thesis as it explores the colloidal calcium phosphate binding of the caseins within the micelle, the internal structured model will be used in all references to the casein micelle (Figure 2.2).

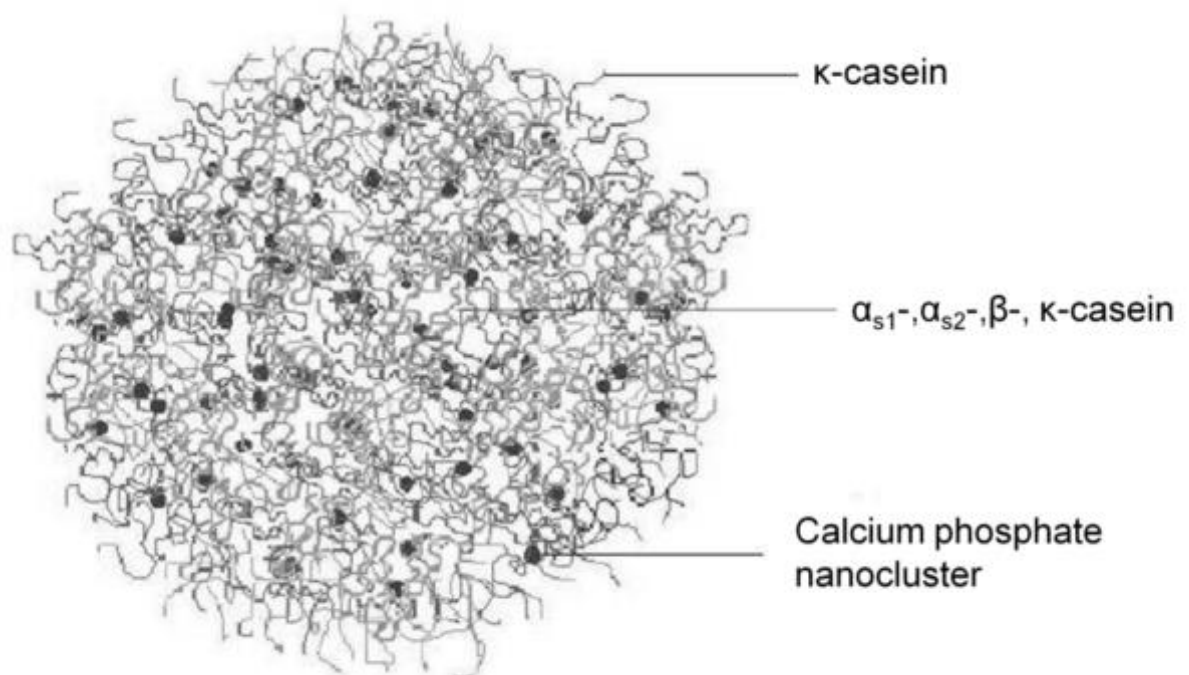


Figure 2.2. Casein micelle internal structure model (De Kruif and Holt, 2003)

#### 2.2.4 $\alpha$ caseins

$\alpha$  caseins have been broken into two distinct groups within the casein micelle,  $\alpha_{s1}$  and  $\alpha_{s2}$  casein. The  $\alpha_{s1}$  casein has been found to be a large proponent of the total casein fraction found in milk, contributing up to 40% of the total casein fraction (Bijl et al., 2014).  $\alpha_{s1}$  casein has two hydrophobic tails, and in low calcium solutions or at high ionic strengths, the casein has the ability to link end to end with itself to create polymer like chains. When not under these conditions, the casein is useful in linking the other caseins within the interior of the micelle to produce “wormlike chains” and provide structure and stability to the interior (McMahon and Oommen, 2008; Horne, 1998).  $\alpha_{s2}$  casein constitutes only 10% of the micelle and is found in the micelle as a disulphide linked dimer (Fox, 2003). This casein is the most hydrophilic of all of the casein types because of the large positive charge found on its C terminal end (Swaisgood, 1993; Farrell et al., 2004).

#### 2.2.5 $\beta$ casein

Beta casein is found within the casein micelle and is thought to have a structure that is almost an exact opposite of that of  $\kappa$  casein.  $\beta$  casein has a hydrophobic N-terminal end that allows it to connect with both  $\alpha$  caseins and  $\kappa$  caseins. This ability raised questions as to where the  $\beta$  casein is found in the molecule (Horne, 1998). However, it has been determined by Dalglish (1998) through centrifugal separation that  $\beta$  casein is indeed found in the interior of the micelle. This was thought to be at odds with the behavior of  $\beta$  casein at low temperatures, where it was found to dissociate out of the micelle and into a separate fraction. Further research into the structure of the micelle and behavior of  $\beta$  casein suggest that at low temperatures, the hydrophobic bonds that

hold the casein in the micelle with the  $\alpha$  casein are at their weakest and dissociate to allow the  $\beta$  casein out of the micelle core and is permitted through the hairy layer of  $\kappa$  casein at the surface of the micelle (Dalgleish, 1998; Walstra, 1990; Fox and Brodtkorb, 2008). This is possible because the  $\kappa$  casein is not a tightly knit coat around the micelle, but rather a hairy layer with gaps and holes.  $\beta$  casein is also the most hydrophobic of the caseins, therefore the most susceptible to dissociation when the hydrophobic bonds are weakened (Farrell et al., 2004). The dissociation of  $\beta$  casein out of the micelle is a temperature dependent reaction, and is reversible when temperature is increased.

$\beta$  casein was found to be crucial for the production of cheese. Increasing the amount of  $\beta$  casein in cheese milk was found to also increase the rennet clotting time and increase the curd firmness of coagulated milk (Jimenez-Flores and Richardson, 1988; Huppertz et al., 2006). The more firm curd is of interest in both increasing the cheese yields of a product and also manipulation of the cheese texture.

#### 2.2.6 $\kappa$ casein

To many, the most well understood of the individual caseins is  $\kappa$  casein.  $\kappa$  casein is unique from other caseins in that it contains both hydrophobic and hydrophilic tails. The hydrophobic tail is found at the N-terminal and the C-terminal is hydrophilic (Walstra, 1990; Horne, 1998). These tails make  $\kappa$ -casein the most important component in determining the outer structure and stability of the casein micelle. The inner caseins ( $\alpha_{s1}$ ,  $\alpha_{s2}$ , and  $\beta$ ) are hydrophobic, and choose to connect with each other and repel the outer solution. It is the function of the hydrophobic N-terminal to connect to the other caseins, and the function of the hydrophilic C-terminal to protrude from the micelle as “flexible hairs”. These hairs are thought to connect the micelles to the aqueous phase of

the milk serum and suspend them, creating the colloidal solution of milk (Creamer et al., 1998; Walstra, 1990; Karlsson et al., 2007). While the  $\kappa$  caseins are integral in the colloidal dispersion of the micelle in milk, they do little to create the structure within the molecule. As the only calcium insensitive casein and the only casein lacking a phosphoserine cluster, it is the least likely of the casein types to bind with the CCP (Dalglish, 1998).

K-casein is crucial in the cheese making process. Disrupting the  $\kappa$  caseins, either by removal or cleavage, causes large changes in the micelle steric stability. Cleavage with chymosin produces para-  $\kappa$  casein. This insoluble casein remains at the surface of the micelle, but rather than repelling other micelles, causes them to aggregate in the presence of  $\text{Ca}^{2+}$ , forming the coagulated curd mass in cheese (Creamer et al., 1998). Removal of  $\kappa$ -caseins from the micelle has successfully been done by increasing pH above 6.7 and increasing temperature (Anema, 2007).

#### 2.2.7 Colloidal Calcium Phosphate

The dry matter of the casein micelle is found to consist of 94% protein and 6% minerals (Horne, 2006). The inorganic constituents form colloidal calcium phosphate, commonly referred to as CCP. Of the 6% that is minerals, 37% is calcium and 50% is phosphate, with magnesium and citrate making up the rest of the mineral constituent (Guo et al., 2003; Holt et al., 1989). The exact percent composition is largely dependent on the individual animal, as well as the breed, season, and stage of lactation (Gaucheron, 2005). Several different functions of the CCP have been suggested within the micellar structure, with the most generally agreed upon being the function of CCP as nanoclusters of a “cement” sequestered between the  $\alpha_{s1}$ ,  $\alpha_{s2}$  and  $\beta$  caseins within the interior of the

micelle (Horne, 2006). The CCP is responsible for the cross linking of the micelles, binding mainly to the phosphate groups of phosphoserine residues. Because of this, the highest binding is found to  $\alpha_{s1}$  caseins, and the least binding to  $\kappa$  casein (Gaucheron, 2005).

The CCP forms bonds by creating bridging between the caseins, causing the micelle to remain in tight formation. Because of this, the equilibrium of CCP within the colloidal solution is extremely important to the structure and existence of the casein micelle (Lucey, 2002; Holt et al., 1989; Bijl et al., 2013). The equilibria between the micellar CCP and the dissolved calcium phosphate in the milk serum is heavily weighted towards the micelle, with a 2/3 to 1/3 balance. This equilibria positions 2/3 of the calcium phosphate in the casein micelle and 1/3 in the serum phase (Walstra, 1990). Because the CCP is so vital in the structure of the micelle, the casein micelle can easily be dissociated by disrupting the equilibrium balance, forcing the CCP out of the micelle and into the solution, or bonding it with another substance. The CCP equilibrium is largely dependent on pH and temperature (Gaucheron, 2005; Bijl et al., 2013). If the equilibrium is disrupted and CCP is pulled out of the micelle, the micelle dissociates and caseins separate out into the serum phase of milk (Fox and Brodkorb, 2008). The dissociation of the micelle can be determined by a decrease in turbidity of the milk, changing the solution from white to a clear or opaque yellow liquid (Odagiri and Nickerson, 1965).

### 2.3 Chelators

Chelators are a group of compounds that bind very tightly with a metal. In the instance of the colloidal solution of milk and specifically the casein micelle as is pertinent to this research, the role of a chelator is to bind with the calcium in the colloidal calcium



phosphate. Therefore, the only chelators focused on in this thesis will be calcium chelators. When the chelator binds with the calcium in the serum, the CCP equilibria is disrupted. To correct this and bring the system back to equilibrium, calcium is pulled out of the micelle. As this depletes the CCP, the structure of the micelle is broken down and caseins are released from the micelle (de Kort et al., 2011; Gaucheron, 2005).

Chelated milk products are thought to exhibit several signs that the casein micelle has been disrupted. The largest sign of disrupted casein systems include a decrease in turbidity of the milk system. As the micelle is dissociated into smaller pieces, the light scattering ability decreases, causing a decrease in the turbidity (Fox, 2003). It has also been found that chelating milk samples results in reduced sedimentation after ultracentrifugation. Because casein micelles are found in the sedimented product in centrifugation, this reduction of sediment also suggests an association between chelation and disruption of casein micelles (Morr, 1967).

Chelators that are commonly used on casein micelle systems are citrates, phosphates, and polyphosphates. All of these chelators have high affinity for calcium (Gaucheron, 2005). De Kort has noted that unlike the temperature dependent separation of  $\beta$  casein from the micelle which is reversible, chelation with citrates, phosphates, and polyphosphates is thought to create an irreversible disruption of the casein micelle. It is unclear, however, if the individual casein fractions, once separated out, will reaggregate in a different structure, because  $\alpha_{s1}$  and  $\beta$  casein, specifically, have a predisposition to self-aggregate via hydrophobic interactions (de Kort et al., 2011; Mittol, 2014).

The usage of mineral chelators to disrupt the casein micelle has been studied widely. Odagiri and Nickerson (1965) studied the effect of calcium chelators on the

change in turbidity and amount of bound calcium in milk systems and saw a decrease in turbidity upon usage of hexametaphosphate, oxalate, citrate, or EDTA. Similar work evaluating the turbidity decrease of milk protein concentrate was done by Mizuno and Lucey (2005), noting that trisodium citrate and sodium hexametaphosphate both caused a decrease in the turbidity and amount of bound calcium and phosphorous. Kaliappan and Lucey (2011) also studied the usage of chelator mixtures and found that the usage of a combination of citrate and phosphate salts caused differences in the physicochemical properties of the micelle. Lastly, de Kort (2009, 2011, 2012) has contributed largely to the knowledge of this system and the usage of calcium chelators, studying the effect of a variety of phosphate and citrates as chelators and their effect on the physical changes of the system and heat induced changes.

### 2.3.1 Citrates

In terms of chelation, the main differences between citrates and phosphates include the final state of the bonding reaction product. Citrates chelate calcium out of the micelle and are then dissolved into the soluble milk serum as a stable calcium citrate complex (Mizuno and Lucey, 2005; de Kort, 2012; Morr, 1967; Vujicic et al., 1968).

Trisodium citrate (TSC) is commonly used within the dairy industry, specifically within processed cheese as an emulsifying salt because it will chelate the calcium within the micellar system but will not bind to the caseins. No gel formation was found within systems treated with sodium citrate because the chelator does not cause the caseins to crosslink upon dissociation (Mizuno and Lucey, 2005). De Kort found that trisodium citrate had a lower affinity for calcium ions than sodium hexametaphosphate or sodium phytate, which lead to less micelle dissociation in samples treated with TSC (de Kort et

al., 2011). Dissociation of the casein micelle was found to begin at levels as low as 35 mEq/L but did not quickly progress, and much of the casein micelle is not yet dissociated at levels as high as 100 mEq/L (de Kort et al., 2012).

### 2.3.2 Phosphates

Whereas citrates chelate the calcium and form a product outside of the casein micelle, many phosphates and polyphosphates bind to the calcium and remain attached to the casein micelle (Guo et al., 2003; de Kort et al., 2011). Phosphates are used in industry to improve the heat stability of milk during treatment and to increase the stability of heat treated milks during storage, as well as to increase the water transfer during drying and reconstitution (Gaucher et al., 2007). Guo (2003) found that adding monophosphate to a casein enriched system caused protein separation at levels as high as 97%, proving phosphates to be useful as a chelator in the separation of casein from the milk serum. Polyphosphates are more prone to creating a gel matrix than other chelators. Because they carry a strong negative charge, phosphates not only bind the positively charged calcium ions in the serum, but can also bind to the positively charged amino acids of casein residues (de Kort et al., 2012; Anema, 2015).

Sodium hexametaphosphate is a commonly used sequesterant and food additive as well as a highly effective chelator that has been the focus of many studies in the dairy industry. Kaliappan and Lucey (2011), Odagiri and Nickerson (1965), Anema (2015), and de Kort et al. (2009) all reported that sodium hexametaphosphate successfully decreased the amount of colloidal calcium phosphate in the micelle when the chelator was used alone or in conjunction with other mineral chelators. De Kort (2012) suggested that the distribution of six negative charges around sodium hexametaphosphate would

allow the chelator to bind with both the calcium ions and casein residues, creating a gel matrix. Mizuno and Lucey (2007) also found that the cross linking and gel matrix formation was caused by a decrease in the charge repulsion and the ability of a polyphosphate to help create hydrophobic bonds within the caseins. Commonly seen in systems treated with sodium hexametaphosphate is an increase in viscosity due to the swelling of the micelles as they cross link with each other to form larger micellar systems as the calcium is sequestered out of the system (de Kort et al., 2011).

### 2.3.3 Ethylenediaminetetraacetic Acid

There has been extensive research into the area of ethylenediaminetetraacetic acid (EDTA) and the changes it causes in skim milk systems. EDTA is a strong chelator, with a charge of negative four and the ability to chelate two calcium ions per molecule. This makes it a stronger chelator than citrates, but weaker than sodium hexametaphosphate, which has a charge of negative six and the ability to chelate three calcium ions per molecule (Udabage et al., 2000). Lin found that the dissociation of the micelle due to EDTA addition was a very rapid process, taking less than a few seconds. This is different from the chelation process of citrates, that has been thought to be a slow process, potentially because of the larger negative charge of EDTA and the ability to chelate more calcium per molecule (Lin et al., 1972). Varying levels of EDTA were added to milk systems, with low levels suggested as 5mM (1.25 mEq/L) and high levels at 20 mM (5 mEq/L). Udabage (2000), Griffin (1988) and Tessier and Rose (1957) all saw the same decrease in pelleted casein, or micellar casein, within these usage levels, with the higher levels of EDTA addition resulting in a smaller micelle diameter. At 5mM addition of EDTA, 5% of micellar casein was released, and with an increase to 10mM EDTA, 30%

of micellar casein was released. Marchin also saw that EDTA caused a dissociation of casein micelles, but countered that a certain critical percentage of calcium must be chelated out before dissociation and size reduction of the casein micelle could be noticed. Marchin et al. (2007) noted that calcium chelation by EDTA showed that although the casein micelle structure was changed there was no indication that EDTA caused creation of small particles, cross linking, or submicelles in solution.

Since EDTA has been extensively studied, it was excluded from this research after pilot study work. Ward, Lin, and Griffin all studied the partitioning of individual caseins within the supernatant upon chelation and separation in milk. Ward (1997) determined that the addition of EDTA to milk resulted in a 10% increase in  $\beta$  casein in supernatant in high heat milk. Lin et al. (1972) found that at low temperatures,  $\beta$  casein and  $\kappa$  casein were released into the supernatant and Griffin (1988) saw no change in the individual casein composition of the supernatant of centrifuged milk treated with EDTA at room temperature. Differing chelation methods, separation conditions, and sample temperatures may have all played a role in the varying results from these studies. As is, there was no conclusive trend found in the partitioning of the individual caseins, but all studies agreed that the addition of EDTA resulted in the chelation of calcium and the dissociation of the casein micelle to some extent.

## 2.4 Separation

When casein micelles have been treated with a chelator to disrupt the micelle and create a solution of individual caseins, it may be possible to separate out these casein fractionates individually in order to take advantage of the unique functional properties of the individual caseins. Several separation techniques are used in industry to separate milk

components, including membrane filtration, centrifugal separation, and coagulation based separation techniques. Separation methods can be further classified based on the separation principle governing them. This breakdown could classify methods into three categories – size exclusion, solubility, and charge.

#### 2.4.1 Size Based Separation

##### 2.4.1.1 Membrane Filtration

Membrane filtration is a unit operation widely used within the dairy industry as a separation and concentration technique. Using a membrane filtration system under pressure allows separation of very small particles, including those the size of fat globules and casein micelles (Kromkamp et al., 2007). Figure 2.3 shows the four types of filtration and what each filters out. These filtration categories include microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO). Microfiltration and ultrafiltration have become commonly used in the dairy industry to create high protein concentrates, fractionate out fat globules, and separate out proteins in the milk serum. To create a protein intense product, the filtration system takes skim milk and filters out even greater amounts of the smaller molecules into a permeate stream using diafiltration. The retentate, which is too large to pass through the membrane, is therefore a milk rich in proteins (Kethireddipalli et al., 2010). The concentrated casein protein system can then be used in standardizing milk, making a customized protein level for cheese milk, or creating dried casein powders (Brans et al., 2004). This system can be intensified and adapted by varying the membrane size and pressure levels in order to separate out the fractions of interest. Two commonly used systems include cross flow filtration in which the sample is filtered as it flows parallel to the surface of the membrane and dead-end

filtration, which sample flows directly towards the membrane (Figure 2.4). Membrane filtration is desirable in industry because it is a relatively low energy process that does not require additives or additional steps in order to provide high levels of separation (Saxena et al., 2009).

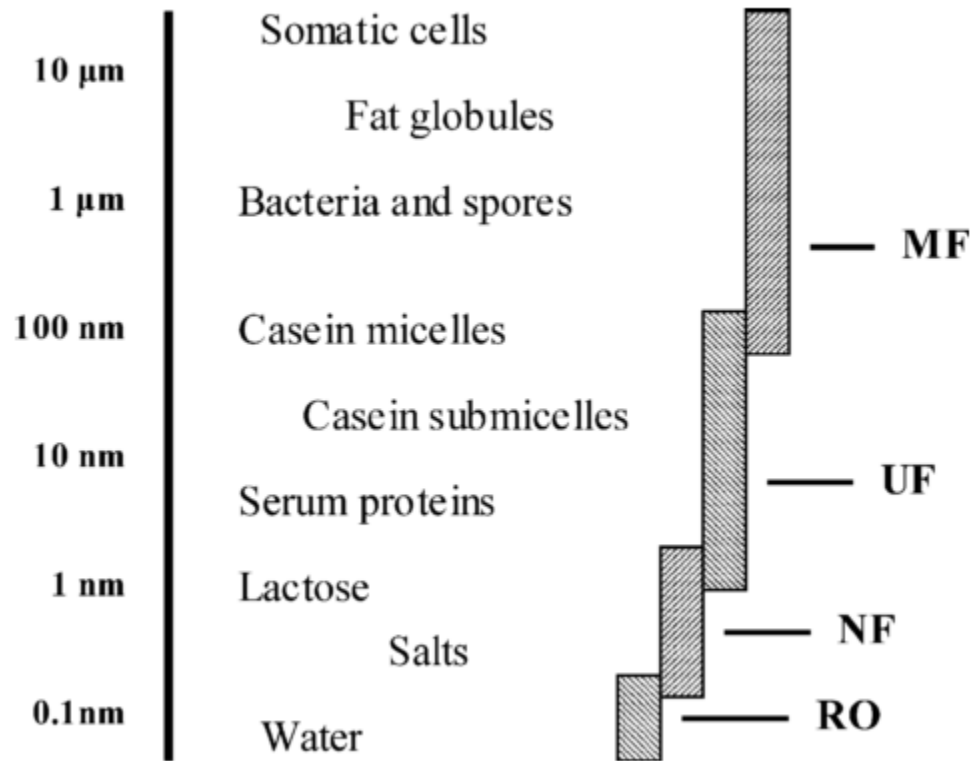


Figure 2.3. Membrane filtration classifications (Brans et al., 2004)

Although membranes are implemented in a variety of ways in the dairy industry, they are limited in their size separation ability. In order to separate out molecules, they must vary in size by a factor of at least ten (Zydney, 1998). While this is beneficial in separating out fats, proteins, and water—molecules with large size differences—from each other, more work is being done to determine the feasibility of membranes as a

process tool to separate out molecules of similar size, including the use of high performance tangential flow filtration and electrically charged membranes (Zydney, 1998; Pouliot, 2008).

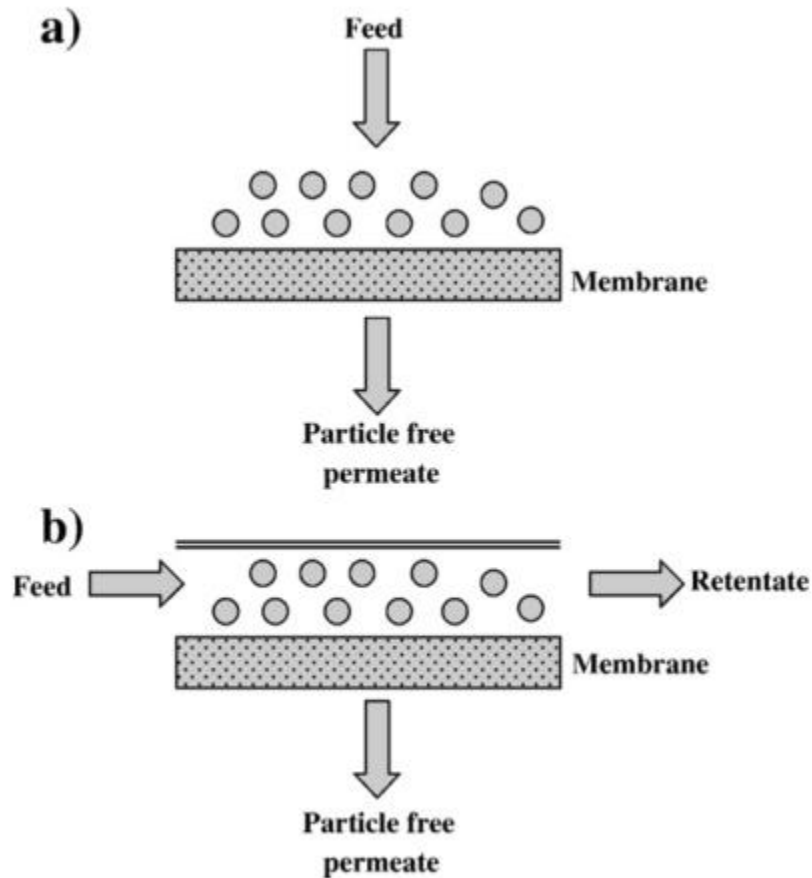


Figure 2.4. Example of a) dead end filtration and b) cross flow filtration (Saxena et al., 2009)

#### 2.4.1.2 Centrifugation

While membrane filtration can also be used for fat separation, the most widely utilized way of cream separation is centrifugation (Brans et al., 2004). Centrifugation provides separation on the basis of particle size and density. When centrifugal force is



applied, molecules with densities higher than that of the solvent will fall out of solution and create a solid pellet. On the laboratory scale, the use of centrifugation allows for the separation of casein micelles from whey proteins and smaller molecules. Because casein micelles are so large, they are sedimented out by centrifugation while the whey proteins stay in the serum, also known as the supernatant (Fox, 2003).

Centrifugal force is measured in relative centrifugal field, a multiple of gravitational force (xg) and is calculated based on the radius of rotation and the rotations per minute of the sample. A wide variety of centrifugal force and times have been used in pelleting casein from milk samples. Anema (2007) reported that force as low as 25,000xg were “sufficient to deposit the casein micelles in unheated milk” , and suggested that the lower force may be more selective in only separating out the casein micelles. The low levels of force is sufficient to pellet the large casein micelles, but not harsh enough to allow additional serum phase aggregates of mid-size to precipitate. Other studies have taken more harsh approaches, such as those of Gaucheron (2005) and Morr (1967), who used speeds of 100,000xg and 144,000xg for 60 mins and 120 minutes, respectively. It was confirmed by Morr and Swenson (1973) in a study on yield and casein and lipid composition as a function of centrifugation time that casein micelle sedimentation is complete at times of less than one hour.

#### 2.4.1.3 Gel Permeation Chromatography

Gel permeation chromatography (also referred to as gel permeation filtration) is a third method of separation based on particle size. Gel permeation is desirable because it is not at all influenced by the elemental make-up of the substance, but rather only its molecular weight. This method is possible to create fractions ranging from molecular

weights of 18 to 1,000,000 Daltons and can be used in a variety of ways, from isolating a specific molecular weight compound to creating a multitude of fractions with different molecular weights from the same main substance (Altgelt and Moore, 2013). This separation technique uses a column packed with porous gel beads of a various size. The sample in solvent is applied to the column. The small particles are able to permeate the gel, but the large particles cannot. Therefore, the large particles stay in the solvent and are quickly eluted from the column while the smaller particles must travel through the gel particles and are eluted more slowly from the substance. A diagram of this size exclusion method is seen below in Figure 2.5. A pointedly beneficial aspect of this method is that the process is semi continuous. As one run is finishing running through the column, the next run can already be beginning to travel through the column (Altgelt and Moore, 2013).

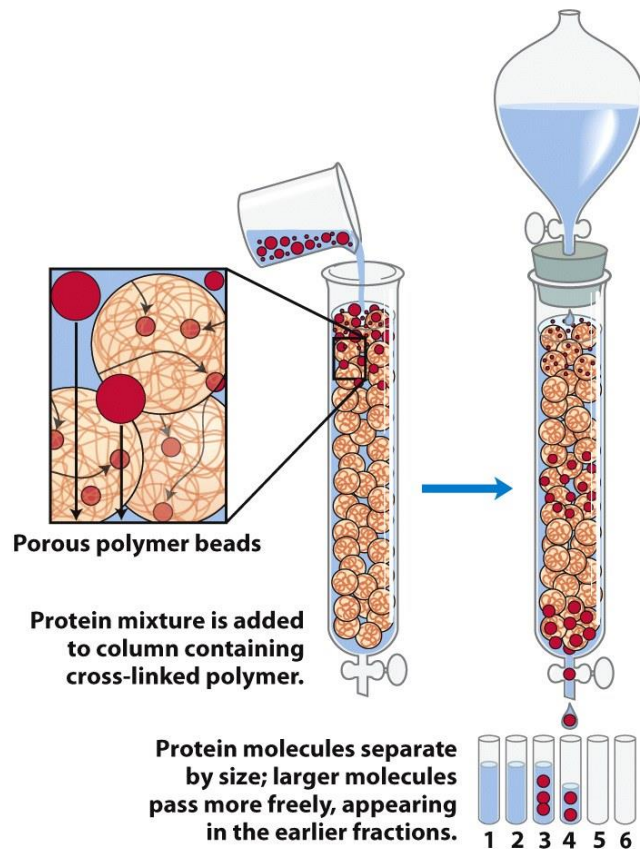


Figure 2.5. Example of gel permeation chromatography (Lehninger et al., 2008)

While this method of separation is widely used within the biopharmaceutical industry, and work has been done to separate out the whey protein fraction in dairy applications, it is not widely used in industrial or laboratory operations in the dairy industry (Fox, 2003). Several, including Pederson and Shimazaki and Sukegawa, have reported success in separating out the various whey proteins using Fractogel and Sepharose columns. Downey and Murphy successfully separated  $\beta$  casein out of the casein micelle using gel filtration, with approximately 60% of the  $\beta$  casein being removed. This was done without any disruption of the micelle or reduced size of the micelle. The success of this separation is thought to be largely due to the low temperatures (4°C) used in the separation, as  $\beta$  casein is known to separate out at low

temperatures due to the decreased hydrophobic bonds (Downey and Kearney, 1970). This success has not transferred into large industrial acceptance and usage of the separation method or in usage as a casein separation method (Pedersen et al., 2003; Shimazaki and Sukegawa, 1982).

## 2.4.2 Solubility

### 2.4.2.1 Heat Coagulation

The usage of heat based coagulation has long been used in the cheese industry as a separation method of curd and whey. While in these cases an acidulant or starter culture is most commonly also used, the use of heat to create a firm curd and expel whey is a common practice. In these situations, the coagulum holds all the caseins, still in the micelle, while the whey proteins are separated out in the whey. When in its natural, unaltered form, casein micelles are fairly resistant to heat treatment (O'Connell and Fox, 2000). Yet heat induced coagulation is apparent in milk systems when higher temperatures are reached. This is partially due to the denaturation of whey proteins at high temperatures.

When  $\beta$  lactoglobulin is denatured, it can result in sulphydryl-disulphide interaction between  $\beta$  lactoglobulin and  $\kappa$  casein (Fox and Brodkorb, 2008). The interaction between the  $\beta$  lactoglobulin and  $\kappa$  casein caused the micelles to be pulled closer together and creates the coagulum. Koutina found that as milk temperature is increased, the amount of free calcium and phosphorus in the serum decreases, reducing electrostatic repulsion and causing aggregation at higher pH (Koutina and Skibsted, 2015). The interaction between  $\beta$  lactoglobulin and  $\kappa$  casein and created coagulum suggests a possibility in creating a  $\kappa$  casein poor supernatant in heated, chelated samples.

With the individual caseins no longer in micellar form, the  $\kappa$  casein and  $\beta$  lactoglobulin would still interact, but the  $\alpha$  casein and  $\beta$  casein would no longer be involved in the coagulum, leaving them free in the supernatant.

However, it has also been shown that the reduction of the colloidal calcium phosphate in a milk system results in an increase in heat stability, or lack of apparent coagulum. This has been found when phosphates and or citrates are added (Muir, 1984; de Kort et al., 2012). De Kort (de Kort et al., 2012) studied the heat coagulation and heat stability of milk treated with chelators extensively. It was found that there is a strong link between temperature and pH in the ability of milk to coagulate. As temperature is increased, the pH required for coagulation is higher, and vice versa (Vasbinder et al., 2003).

#### 2.4.2.2 Acidification

Milk naturally has a pH of approximately 6.7, and casein micelles exist with a net negative charge in solution. When the pH of the solution is dropped to below the isoionic point of the caseins, the electrostatic repulsion is weakened and a coagulum is formed, causing the caseins to aggregate (Crujisen, 1996; Koutina and Skibsted, 2015). The isoionic point of each of the individual caseins is listed in Table 2.2 with reference variant for each casein in bold. The calcium concentration of the solution is vital to the ability of the solution to begin pH based coagulation. As acidification occurs, the serum gains a larger negative charge, causing solubilization of the calcium phosphate and a disintegration of the casein micelle (Muir, 1984). Walstra (1990) suggested that this decrease in free calcium ions and resulting increased negative charge of the serum also may cause the casein chains to “curl up” and increase rate of coagulation. Crujisen (1996)

suggested that the calcium concentration required is equivalent to the phosphoserine residues of the casein micelles. Acidification is common in the dairy industry and used in many applications including the manufacture of yogurt, cottage cheese, and as a beginning step in some cheesemaking processes (Chardot et al., 2002).

Singh et al. (1996) found that at room temperature, non-pelleted casein would peak at a pH of approximately 5.5, and then decrease as the pH was dropped closer to the 4.6 isoionic pH of the casein micelle where the casein would coagulate and remain as a pellet. This was similar to the findings of McMahon et al. (2009), which stated that milk started to gel at a pH of 5.2-5.4 and was completely gelled by a pH of 4.8. This was proposed as part of a 3 phase model of acid based gelation. Dalgleish and Law (1988) extensively studied the effect of pH value on the dissociation and separation of casein micelles, and found that  $\beta$  casein and  $\alpha_{s1}$  casein release varied based on pH, while  $\kappa$  casein and  $\alpha_{s2}$  casein were independent of pH. Dalgleish and Law did not see the precipitation of casein from the micelle as concurrent to the isoionic pH of the individual caseins, and all dissociations were largely temperature dependent.

Table 2.2. Isoionic pH of casein protein variants adapted from Swaisgood (1993) and Fox (2003) with reference variant of each casein bolded

Protein	Isoionic pH <sup>1</sup>
$\alpha_{s1}$ -CN	
<b>A-8P</b>	<b>4.94</b>
B-8P	4.94
C-8P	4.97
D-9P	4.88
$\alpha_{s2}$ -CN	
A-10P	5.45
<b>A-11P</b>	<b>5.37</b>
A-12P	5.30
A-13P	5.23
$\beta$ -CN	
A <sup>3</sup> -5P	5.07
<b>A<sup>2</sup>-5P</b>	<b>5.14</b>
A <sup>1</sup> -5P	5.22
B-5P	5.29
C-4P	5.46
$\kappa$ -CN	
<b>A-1P</b>	<b>5.61</b>
B-1P	5.90

<sup>1</sup>Calculated as for charge at pH 6.6, but  $Z_H = 0$  at the isoionic pH.

### 2.4.3 Ion Exchange Chromatography

Ion Exchange chromatography is an effective and widely used separation technique in laboratory and industrial separations. The technique utilizes the ability of a charged column resin to bind with components of an opposite charge as they are applied to the column. The column is flushed with a mobile phase salt gradient which competes for binding sites and elutes the proteins off of the column. This method is effective in separation because the elution is based on the ability of the molecule to bind tightly with the resin. Molecules with the lowest binding ability or weakest ionic interactions will elute first, and those with stronger binding ability will require higher salt concentrations, thus eluting later. Two types of ion exchange chromatography are used – anion exchange

chromatography, which uses a positively charged resin and binds negatively charged molecules, and cation exchange chromatography (Figure 2.6), with a negative charge resin that binds positively charged molecules (Xu, 2005).

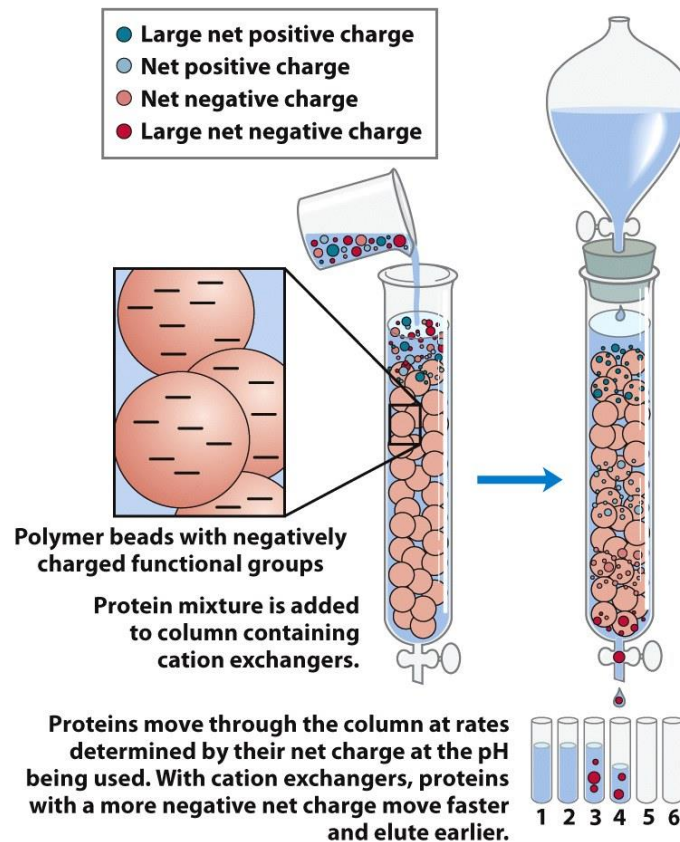


Figure 2.6. Example of cation exchange chromatography showing a positively charged resin binding negatively charged molecules (Lehninger et al., 2008)

Whey protein separation work has been done extensively on ion exchange columns. Casein separation has also proven feasible by Hollar et al. (1991) and others using both anion exchange and cation exchange fast protein liquid chromatography. Cation exchange chromatography gave good, timely separation and easy quantification and was the preferred method of Hollar. Davies and Law (1977) also worked extensively



in this area and preferred the usage of anion exchange chromatography using a DEAE cellulose column, which they felt had better recovery of protein from the column with recovery values between 94% and 98% of sample.

While ion exchange chromatography provides a useful lab scale technique, the application of ion exchange theory to membranes has created a larger impact in the ability to separated proteins industrially. Ion exchange membranes are a membrane filtration technique that applies negative or positively charged groups to the membrane backbone, either chemically or physically. This allows separation of solutions based both on particle size and particle charge, making it possible to achieve more precise separations (Xu, 2005). The usage of ion exchange membranes has been extensively studied, including work by Plate, Lu, Wolman, Recio and Visser, as a way to separate whey proteins both lab scale and industrially, specifically as a way to separate lactoferrin (Plate et al., 2006; Lu et al., 2007; Wolman et al., 2007; Recio and Visser, 1999).

## 2.5 Summary

Casein micelles are a complex and highly studied system. They are comprised of four separate caseins –  $\alpha_{s1}$ ,  $\alpha_{s2}$ ,  $\beta$ , and  $\kappa$  casein, as well as the minerals of calcium and phosphate. When a chelating agent such as a citrate, phosphate, or polyphosphate are added to milk systems, the colloidal calcium phosphate is bound to the chelator and pulled out of the micelle. This disruption of the micelle causes the structure to collapse and the caseins are thought to become separated from each other. The disruption of the casein micelle and separation of caseins into solution is an excellent opening for research into the possible exploitation of the unique individual functional properties of the individual caseins. Once micelles are disrupted by chelators, the entire solution may be

able to undergo separation to create individual fractionates of caseins. Many separation avenues are possible in dairy technology, including separation based on size, solubility and charge. The further functionalization of the casein micelle fractionates will allow a wide range of possibilities to be opened within the dairy ingredient industry, allowing the growing protein markets to continue to explore new products and trends.

### 3. JUSTIFICATION AND HYPOTHESIS

The ability to explore the functionality of the caseins individually may be of great interest to the dairy industry because of the previously mentioned possibility to functionalize and leverage the usage differences between the caseins. The separation of  $\beta$  casein from the micelle using temperature changes has been well documented, as has the ability to separate the intact casein micelle from the serum using common separation techniques. However, the effect of the pretreatment of milk using calcium chelators on the separation/partitioning of individual caseins has not been widely studied and reported on.

Therefore, this thesis project seeks to use multiple separation techniques (membrane filtration, ultracentrifugation, heat, and acidification) on milk treated with calcium chelators in order to evaluate the protein/casein fractionation within the disrupted system.

Based upon the review of literature, the following hypothesis were formed:

1. The type of calcium chelator (phosphate vs citrate) and the level of chelator used will impact the amount of dissociation of the casein micelle.
2. The partitioning of the individual caseins from the disrupted casein micelle will be effected by the type of chelator used and level of chelator used within the separation method.
3. The casein partitioning can be manipulated by changes within the separation method used (changes in force, pH, and temperature).

The objectives of this study were then:

1. To disrupt the casein micelle by calcium chelation and to show the differences in dissociation of the casein micelle using particle size analysis, total protein analysis, and turbidity analysis
2. To determine feasibility of common separation methods on chelated milk systems to create different partitions of individual caseins

## 4. MATERIALS AND METHODS

### 4.1 Experimental Design

For each separation method (“separation study”), a completely randomized design was followed. Each study contained a different number of separation treatments and will be discussed separately within individual sections, with a general treatment model shown below:

$$y_{ijkt} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + (\alpha\beta\gamma)_{ijk} + \varepsilon_{ijkt}$$

where:

$y_{ijkt}$  represents the  $t^{\text{th}}$  response of the  $i^{\text{th}}$  level of chelator type,  $j^{\text{th}}$  level of chelator level, and  $k^{\text{th}}$  level of separation variation

$\mu$  represents the overall mean response

$\alpha_i$  represents the main effect of the  $i^{\text{th}}$  level of chelator type

$\beta_j$  represents the main effect of the  $j^{\text{th}}$  level of chelator level

$\gamma_k$  represents the main effect of the  $k^{\text{th}}$  level of separation variation

$(\alpha\beta)_{ij}$  represents the interaction effect of  $i^{\text{th}}$  level of chelator type and  $j^{\text{th}}$  level of chelator level

$(\alpha\gamma)_{ik}$  represents the interaction effect of  $i^{\text{th}}$  level of chelator type and  $k^{\text{th}}$  level of separation variation

$(\beta\gamma)_{jk}$  represents the interaction effect of  $j^{\text{th}}$  level of chelator level and  $k^{\text{th}}$  level of separation variation

$(\alpha\beta\gamma)_{ijk}$  represents the interaction effect of  $i^{\text{th}}$  level of chelator type,  $j^{\text{th}}$  level of chelator level, and  $k^{\text{th}}$  level of separation variation

$\varepsilon_{ijkt}$  represents the error on the  $t^{\text{th}}$  response of the  $i^{\text{th}}$  level of chelator type,  $j^{\text{th}}$  level of chelator level, and  $k^{\text{th}}$  level of separation variation, and we assume  $\varepsilon_{ijkt} \sim N(0, \sigma^2)$

Treatment combinations and sample order were randomly assigned using Microsoft Excel random number generator. Testing order for all analytical tests run were also determined in this way.

#### 4.1.1 Treatment Level Determination

SHMP and TSC were used as the chelator types for factor 1, as they represented a citrate and a phosphate. The review of literature, specifically sections 2.3, 2.3.1, and 2.3.2 outline the differences in these two types of chelators and the benefits of exploring both as calcium chelators in these studies.

Three levels of chelator were chosen; 1 mEq, 50 mEq, and 100 mEq. It was decided to measure the chelator in mEq instead of the commonly used mMol as the two types of chelators carry different amounts of negative charges, and these negative charges impact the calcium binding ability. Table 4.6 in section 4.3 shows the corresponding concentrations of each chelator in mmol/L and grams of chelator/grams of skim milk powder. The levels selected were chosen as the lowest level to see change in the strongest chelator, and the highest level to see full dissociation in the weakest chelator, with the midpoint set in between.

A control sample was included for observation only, not as part of balanced design. This control could not be considered part of a balanced design because did not have all factors at all levels, specifically chelator level. It was still considered important to run, however, to ensure that the control sample was acting as was expected of a milk sample being separated and ensure that any variation seen was through the disruption of casein micelles from the chelators, as well as allow a general comparison between chelated and unchelated samples. Control samples were run for each factor 3 treatment level in each study.

#### 4.1.2 Preliminary Power Study

A pilot study was run to determine the approximate statistical power, or likelihood of a difference being detected where there is a difference between the samples. To maximize time and supply constraints, only one variable (chelator type) was varied in the pilot study, with the other two variables fixed at “mid” levels. Only one separation method, ultracentrifugation, was tested. The three chelators were tested at 50 mEq (treatment level 2) and centrifuged at 60,000xg (treatment level 2). These experimental terms are found in Table 4.1, below.

Table 4.1. Definition of terms for experimental design for preliminary power study. SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate, EDTA = Ethylenediaminetetraacetic Acid

Factor 1	
<b>i</b>	<b>Chelator Type</b>
1	SHMP
2	TSC
3	EDTA
Factor 2	
<b>j</b>	<b>Chelator Level</b>
2	50 mEq
Factor 3	
<b>k</b>	<b>Separation Variant (Centrifugal Force)</b>
2	60,000xg

These samples were run in four replicates. Using Minitab 17.0 statistical software, which utilizes the power calculation found in Figure 4.1, it was determined that by running all samples in four replicates, statistical power of 1.000 could be achieved for the responses of protein level,  $\alpha$  casein, and  $\beta$  casein. For the least sensitive response,  $\kappa$  casein, four data replicates gave a power of 0.9209 (Figure 4.2). These statistical powers indicate that there is 100.0% and 92.09% chance, respectively, of correctly detecting a 5% difference in the response (protein level,  $\alpha$  casein,  $\beta$  casein, or  $\kappa$  casein) if a difference truly exists. Based on this study, it was determined that four data replicates would be sufficient.



### Calculating power

#### Degrees of freedom for error

$$k2 = n1 - n2 - n3 - n4$$

#### Non-centrality parameter

$$\beta = n \delta^2 / 2$$

#### Power

$$\text{Power} = 1 - F_{k1, k2, \beta}(v_{k1, k2, 1 - \alpha})$$

#### Notation

$k1 = k - 1$  where  $k$  is the number of levels for the factor with the largest number of levels

$k2 =$  degrees of freedom for error

$n1 =$  total number of runs; number of replicates \*  $\prod (NLi)$  where  $NLi$  is the number of levels for the  $i^{\text{th}}$  factor

$n2 =$  number of replicates - 1 for blocks (if included in the model)

$n3 =$  sum of degrees of freedom for factor terms up to order  $K$

$n4 = 1$  for overall mean

$\beta =$  non-centrality parameter

$\delta =$  maximum difference / standard deviation

$n =$  sample size at each level of the factor with the largest number of levels

$\alpha =$  significance level

$v_{k1, k2, s} =$  inverse CDF at  $s$  for a central F distribution with  $k1, k2$  degrees of freedom

Figure 4.1. Equations used by Minitab 17.0 statistical software for analysis of statistical power using “Power and Sample Size for General Full Factorial Design” command

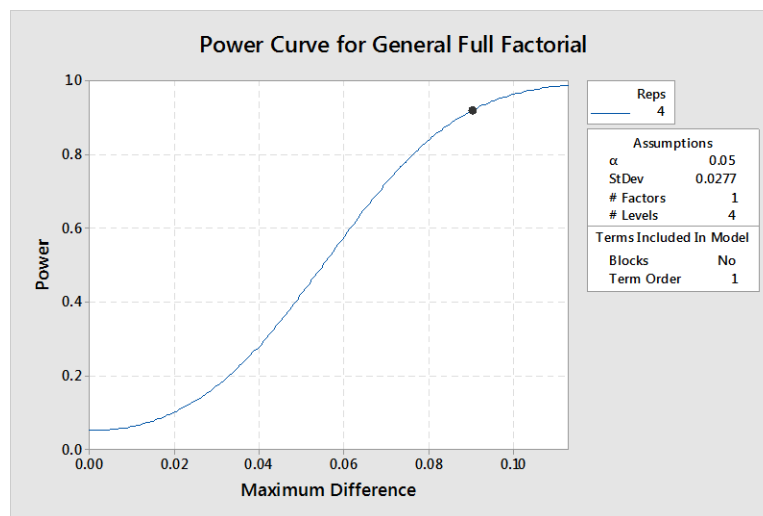


Figure 4.2. Power curve showing that in four data replicates, 92% chance of correctly detecting difference in %  $\kappa$  casein in supernatant of samples

It was determined after this power study that because of restrictions, EDTA would not be included as variable. EDTA has been widely studied as a calcium chelator in milk (see Section 2.3.3) and therefore was eliminated from this research.

#### 4.1.3 Ultracentrifugation Study

This study used a 2x3x3 full factorial design. Table 4.2 outlines the factors of this experimental design. All treatment combinations were run in four replicates. The levels of centrifugal force tested were selected as the lowest level found in literature to see pelleting of casein micelles (30,000xg) and the highest level allowed by the ultracentrifuge rotor (90,000xg), with a midpoint set in between the two extremes.

Table 4.2. Treatment definitions for 2x3x3 full factorial ultracentrifugation study.

SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate

Factor 1	
<b>i</b>	<b>Chelator Type</b>
1	SHMP
2	TSC
Factor 2	
<b>j</b>	<b>Chelator Level</b>
1	1 mEq
2	50 mEq
3	100 mEq
Factor 3	
<b>k</b>	<b>Centrifugal Force</b>
1	30,000xg
2	60,000xg
3	90,000xg

#### 4.1.4 Solubility with Temperature

This study used a 2x3x2 full factorial design. Table 4.3 outlines the factors of this experimental design. All treatment combinations were run in four replicates. The two temperatures tested were chosen as they represent the temperature of HTST pasteurization, and a high temperature potentially seen in temperature abused product, but not outside the capacity of the heating device.

Table 4.3. Treatment definitions for 2x3x2 full factorial solubility with temperature study. SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate

Factor 1	
<b>i</b>	<b>Chelator Type</b>
1	SHMP
2	TSC
Factor 2	
<b>j</b>	<b>Chelator Level</b>
1	1 mEq
2	50 mEq
3	100 mEq
Factor 3	
<b>k</b>	<b>Temperature</b>
1	70°C
2	90°C

#### 4.1.5 Membrane Filtration Study

This study used a 2x3 full factorial design. Table 4.4 outlines the factors of this experimental design. All treatment combinations were run in four replicates. A 10kDa membrane was used for all filtrations.

Table 4.4. Treatment definitions for 2x3 full factorial membrane filtration study.

SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate

Factor 1	
<b>i</b>	<b>Chelator Type</b>
1	SHMP
2	TSC
Factor 2	
<b>j</b>	<b>Chelator Level</b>
1	1 mEq
2	50 mEq
3	100 mEq

#### 4.1.6 Acidification Study

This study used a 2x3x4 full factorial design. Table 4.5 outlines the factors of this experimental design. All treatment combinations were run in four replicates. The pH's used were selected as the represent the isoionic pH of the individual caseins, as explained in section 2.4.2.2.

Table 4.5. Treatment definitions for 2x3x4 full factorial acidification study.

SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate

Factor 1	
<b>i</b>	<b>Chelator Type</b>
1	SHMP
2	TSC
Factor 2	
<b>j</b>	<b>Chelator Level</b>
1	1 mEq
2	50 mEq
3	100 mEq
Factor 3	
<b>k</b>	<b>pH</b>
1	4.94
2	5.13
3	5.37
4	5.61

#### 4.1.7 Data Analysis

The statistical analysis for all experiments was run using Minitab 17.0 statistical software. Analysis for unseparated samples was done using a One-way ANOVA at 7 levels, and full factorial ANOVA as listed above were used in separation studies. Post-hoc comparisons were done using Tukey method, with familywise error rate controlled at 0.05. Control samples without chelator were also analyzed in each study, but control

samples were not included in statistical analysis as a part of the full factorial design. Control samples were chosen not to be analyzed because the large difference between the control and the chelated samples would have been less efficient and made differences between the two chelators appear less significant. As this research seeks to determine the influence of chelator type (citrate vs phosphate) on the dissociation of casein micelles, it was important to use a statistical design that allowed the more accurate analysis of the differences in these two chelators.

#### 4.2 Rehydration of Skim Milk Powder

Samples were rehydrated to a 10% w/v basis using skim milk powder (DairyAmerica, Fresno, CA) in deionized water (50g/500ml). The milk powder was rehydrated on the benchtop in 500ml portions. Water and SMP were added to an 800ml beaker, and stirred at 600rpm for 30 minutes at room temperature ( $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ) using a 1.5 inch stir bar and Corning PC-620D stir plate (Corning Inc., Corning, NY). Rehydrated SMP was then let to sit at room temperature for 4 hours.

#### 4.3 Chelation

Rehydrated SMP was divided into 40ml aliquots in 50ml beakers. A 1 inch stir bar was added and samples were stirred at 300rpm on Corning stir plate. After stirring had begun, the designated amount of Glass H Long Chain sodium hexametaphosphate (ICL Performance Products LP, St. Louis, MO) or sodium citrate dehydrate (Tate & Lyle, Decatur, IL) chelator was added. Samples were allowed to stir at 300rpm for 30 minutes at room temperature ( $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ). Table 4.6 shows the chelator amounts in mEq/L, mM, and g/g SMP. Samples were then covered and refrigerated at  $4^{\circ}\text{C}$  for 18 hours to equilibrate prior to use.

Table 4.6. Chelator Concentrations in mmol/L, mEq/L, and (g chelator/g SMP)

Chelator	1 mEq	50 mEq	100 mEq
Trisodium Citrate	0.33 mM (0.0037g)	16.7mM (0.1833g)	33.4 mM (0.3667g)
Sodium Hexametaphosphate	0.17 mM (0.0009g)	8.4 mM (0.0430g)	16.7 mM (0.0860g)

#### 4.4 Separation Methods

##### 4.4.1 Ultracentrifugation

Room temperature sample was added to a 50 mL centrifuge tube (Thermo Scientific, Rochester, NY) to a total weight of 35.0g  $\pm$ .01g. Samples were then placed in a Beckman Type 28 fixed angle rotor and centrifuged in Beckman L7-35 ultracentrifuge (Beckman Coulter, Brea, CA) at 23°C for 60 minutes. Samples were removed from the rotor and supernatant was decanted off into 50 mL falcon tube. The pellet was washed by adding deionized water to centrifuge tube with pellet to bring weight back to 35.0g and centrifuging for an additional 30 minutes at 23°C. This wash water was then decanted off into the supernatant portion to ensure that soluble proteins were not included in the sedimented pellet portion.

Relative centrifugal force was varied as a treatment level. Centrifugal force was calculated using the rotor radius and the rotations per minute (rpm) in the following equation:

$$\text{RCF} = 1.118 \times 10^{-5} \times r_{\text{max}} \times (\text{rpm})^2$$

RCF = relative centrifugal force (xg)

$r_{\text{max}}$  = rotor radius (maximum) for Beckman Type 28 rotor

rpm = rotations speed in rotations per minute

Maximum rotor radius was used for calculations as the casein pellet forms at the bottom of the tube (Figure 4.3). Rotations per minute were manually set on centrifuge, as was temperature.

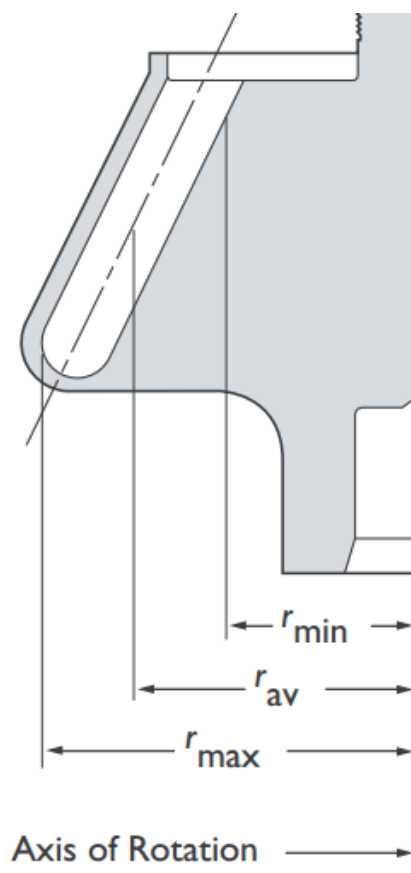


Figure 4.3. Schematic of the placement of  $r_{\text{min}}$ ,  $r_{\text{av}}$  and  $r_{\text{max}}$  on a Beckman-Coulter rotor. The  $r_{\text{max}}$  was used for all calculations as the pellet forms on the bottom on the centrifuge tube (Beckman-Coulter, 2000)



#### 4.4.2 Temperature

Samples were allowed to warm to room temperature and then sample pH was adjusted to 6.3 using 1 N hydrochloric acid. After pH adjustment, 35.0ml of sample were added to a 50 mL falcon tube. These falcon tubes were placed in a preheated ISOTEMP 210 water bath (Fischer Scientific, Pittsburgh, PA) at specified temperatures of 70°C or 90°C. Samples were heated in the water bath for 30 minutes and then removed. They were then centrifuged at 3500xg for 15 minutes to create a firm pellet of the coagulated mass. The supernatant was then decanted off into a 50 mL falcon tube for further analysis.

#### 4.4.3 Membrane Filtration

Membrane filtration was done on the benchtop using an Amicon 8500 stirred ultrafiltration cell unit (Figure 4.4) and polyethersulfone Biomax 10 kDa ultrafiltration discs (EMD Millipore Corporation, Billerica, MA). Unit was assembled on a Corning PC-620D stir plate (Corning Inc., Corning, NY). Membrane disc was inserted into cell unit and the membrane was rinsed with deionized water for 5 minutes at 55psi while stirring at 200 rpm. Flux was measured at this time also.

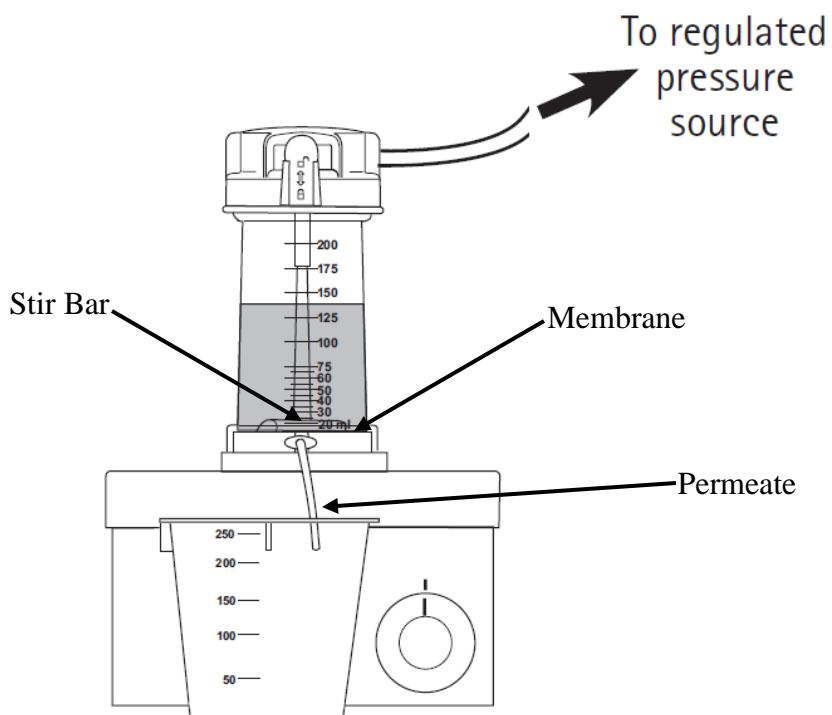


Figure 4.4. Schematic of Amicon stirred cell membrane filtration unit (EMD Millipore, 2015)

To filter, 40 mL of warmed sample (50°C) was added to the stirred cell. Between 200rpm-400rpm stir speed was used, based on the amount of sample in the cell. Speed was monitored to maintain a vortex 1/3 of the way down the sample. Temperature was maintained at 50°C throughout. Permeate was collected and measured until 20 mL, or a 2 times volume concentration ratio, was achieved. Permeate and retentate were then stored in 50 mL falcon tube for analysis. After each filtration, the membrane was rinsed with 0.1 N NaOH for 5 minutes and then rinsed with water until appropriate flux was restored.

#### 4.4.4 Acidification

The pH was manually adjusted in each 50 mL sample by dropwise addition of 1 N hydrochloric acid with stirring at room temperature to sample. The pH was monitored

using Orion 9162BNWP probe and Orion 2 Star pH Benchtop meter (Fischer Scientific, Pittsburgh, PA). The pH adjustment targeted the isoionic pH of the individual caseins to an accuracy of  $\pm 0.02$ . Samples were allowed to equilibrate for 30 minutes after addition of acid, and then rechecked and readjusted to target pH if necessary. Coagulum was centrifuged in Eppendorf 5810 R centrifuge with A-4-62 bucket rotor (Eppendorf, Hamburg, Germany) at 3500rpm (2465xg) for 15 minutes and supernatant was decanted off into 50 mL falcon tube.

#### 4.5 Analytical Methods

##### 4.5.1 Total Nitrogen and Protein

Total Nitrogen was determined using total nitrogen in milk Kjeldahl method - AOAC method 991.20, Block Digester/Steam Distillation method. Three kjeltabs (Fisher Chemical, Pittsburgh, PA) replaced the  $K_2SO_4$  and  $CuSO_4 \cdot 5H_2O$  catalyst solutions, and Kjel-Sorb solution (Fisher Chemical, Pittsburgh, PA) replaced the boric acid with indicator. Samples were digested using Tecator Digester (Foss, Eden Prairie, MN) and distilled using Kjeltec 2200 or Kjeltec 8200 (Foss, Eden Prairie, MN). All samples were run in duplicate and total nitrogen was determined using the following equation.

$$\%N = \frac{1.4007 \times (mL \text{ of } HCl \text{ used}) \times N \text{ HCl}}{g \text{ of sample}}$$

Total nitrogen was converted to % protein by multiplying by a conversion factor of 6.38.

#### 4.5.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)

SDS PAGE was used to separate the individual caseins in each sample for quantification. Reducing buffer stock was made using 95% 2x Laemmli (1970) sample buffer (Bio-RAD, Hercules, CA) and 5% beta mercaptoethanol (SIGMA Life Science, St. Louis, MO). Sample supernatant was diluted 1:20 with prepared reducing buffer stock in 1.5 ml microcentrifuge tubes. Samples were then heated for 5 min at 95°C on Vanlab heat block (VWR Scientific, Inc Radnor, PA) and allowed to cool to room temperature.

Precast 12% acrylamide gel cassettes (Bio-RAD Hercules, CA) were assembled in the Mini-PROTEAN Tetra Cell system. The inner and outer chambers of the cell were filled with 1x Tris/Glycine/SDS running buffer. Sample volumes of 3µL were loaded into the individual wells. The assembled tetra cell system was connected to PowerPAC 300 (Bio-RAD Hercules, CA) and run at a constant 200V for 50 minutes, monitoring for the tracking dye to reach the bottom of the gel.

After electrophoresis, the cassettes were disassembled and the gels were placed in Coomassie Brilliant Blue R-250 staining solution and placed on innova 2000 platform shaker (New Brunswick Scientific, Edison, NJ) at 60rpm for 30 minutes. Stain was then discarded and gels were then destained using destain solution (40% methanol, 10% acetic acid, 50% deionized water) for 10 minutes. Destain was discarded and gels were destained a second time using fresh solution for 12 hours. Gel imaging was then done immediately after using Molecular Imager Gel Doc XR (BioRad, Hercules, CA). These images were analyzed using ImageJ software v1.49 (National Institute of Health, USA) to quantify the intensity of the bands using densitometry. Separate bands were apparent for  $\alpha$  casein,  $\beta$  casein, and  $\kappa$  caseins. These intensities were converted to peaks and the area

of these peaks was given by the ImageJ software. The peak area was then used to calculate the total area and the proportion of each individual casein.

#### 4.5.3 Turbidity

Turbidity was measured using SPECTRAMax Plus 384 (Molecular Devices, Sunnyvale, CA). Three milliliters of room temperature sample were added to 1 cm cuvette. Measurements were taken at 700 nm and performed in duplicate. Turbidity was measured in AU (arbitrary units) at OD<sub>700</sub>. Water was used as a blank reference sample.

#### 4.5.4 Particle Size Analysis

The mean size diameter of supernatant was determined using Coulter LS 230 with Fluid Module (Beckman Coulter, Brea, CA). Prior to running samples, the instrument auto rinse was run. The laser was then aligned, adjustment was made for electrical offsets, and background was measured. Once this calibration was completed, sample loading was measured and room temperature sample was added until obscuration was between 10% and 15%. Polarization Intensity Differential Scattering (PIDS) data was collected to account for the small particle size of the dissociated casein micelles. Pump speed was set at 51 and sample run time was 60 seconds. Beckman Coulter LS Software v 3.29 August 2003 (Brea, CA) automatically converted voltage measurements to particle size diameter distributions using Fraunhofer light scattering model. Measurements of the median particle size ( $\mu\text{m}$ ) were taken in triplicate and a mean average was generated by the software. Auto rinse was run after each sample and offset adjustment, alignment and background measurements were done every hour.

## 5. RESULTS AND DISCUSSION

### 5.1 Unseparated Sample Analysis

Turbidity, pH, and particle size of the samples were monitored after chelating but without any separation techniques executed. The following three sections outline the differences seen with these samples and were compared to unchelated control milk. Chelation of milk samples and analysis of the physical properties of these samples has been widely studied. This work was carried out in this thesis to verify that prior to separation, these samples were behaving in ways similar to that reported in previous studies (Mizuno and Lucey, 2007; de Kort et al., 2011; Udabage et al., 2000).

#### 5.1.1 Turbidity

Because of the size of casein micelles, they have a large impact on the turbidity of milk. The large molecules have greater light scattering ability and cause much of the opacity of milk. Therefore, a decrease in turbidity is a generally well accepted sign of a dissociation of the casein micelle (de Kort, 2012; Fox, 2003). Using a One-way ANOVA with turbidity as response and chelator type and level combinations (at 7 levels) as the factor, it was found that there was at least one statistically significant difference ( $p$  value  $<0.0001$ ) between the chelator/level combinations with regard to mean turbidity. There is strong evidence of a difference in the mean turbidity between all of the treatments except 50 mEq SHMP, 100 mEq SHMP and 100 mEq TSC. These mean turbidity values were so low ( $0.179 \pm 0.02$ ,  $0.102 \pm 0.01$ , and  $0.192 \pm 0.02$ , respectively) that it can be assumed that the calcium has been chelated out of the micelle to a point where the micelle is fully dissociated at this point. While the remaining treatments did not have as low of turbidity readings, there was a decrease in turbidity in all samples, displayed in

Figure 5.1. These results indicate that the chelator is causing micellar dissociation, as the turbidity decrease is coordinated with the increase in the level of chelator added. The faster drop in turbidity as the chelator level is increased seen in the samples treated with SHMP indicated that this is a stronger chelator, dissociating the micelle at lower levels of chelator addition. It is important to note that samples were not diluted before measurement, resulting in the turbidity readings over 1 in the control and samples chelated with low levels of chelator. Turbidity measurements using a spectrometer tend to no longer be linear above levels of 1, so this data is less conclusive than if a dilution had been applied. The decision was made not to dilute the samples however, to maintain consistency with the samples that could not be diluted because of lower initial turbidity readings. Further verification could be done by diluting the highly turbid samples and using a correction factor in these samples. These results, as is, still fall in line with the findings of Mizuno and Lucey (2005) and de Kort et al. (2011).

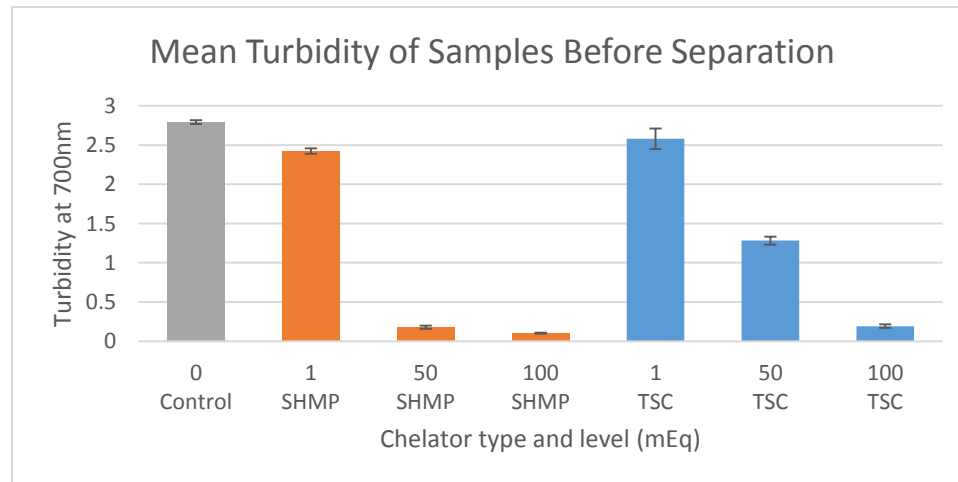


Figure 5.1. Means ( $\pm$ SE) showing the turbidity of the stock (unseparated) chelated samples. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate

### 5.1.2 Particle Size Analysis

Using a One-way ANOVA with particle size as the response and chelator/level combinations (at 7 levels) as the factor, it was found that there was at least one statistically significant difference ( $p\text{-value} < 0.0001$ ) between the chelator/level combinations with regard to mean particle size. The extremely large mean particle size in the samples treated with 100 mEq SHMP ( $11.86 \pm 3.50 \mu\text{m}$ ) stood out compared to the other chelator type/treatment combinations. However, there is an increased mean particle size in the samples treated with 50 mEq SHMP ( $0.658 \pm 0.17 \mu\text{m}$ ) and 100 mEq TSC ( $0.580 \pm 0.14 \mu\text{m}$ ). This is in line with the decrease in turbidity that was also seen in these samples, reported in section 5.1.1. The large particle size seen in the 100 mEq SHMP samples is supported in literature, as many have reported that when treated with a polyphosphate, the caseins form a larger gel matrix in the milk (Mizuno and Lucey, 2007; de Kort et al., 2011). This larger gel matrix is seen as the SHMP binds with the amino acid residues of the casein micelles. The ability of the SHMP to bind with not only the calcium in the micelle but also with the individual caseins separates this chelator from TSC and poses the potential for differences in separation of the micelle.



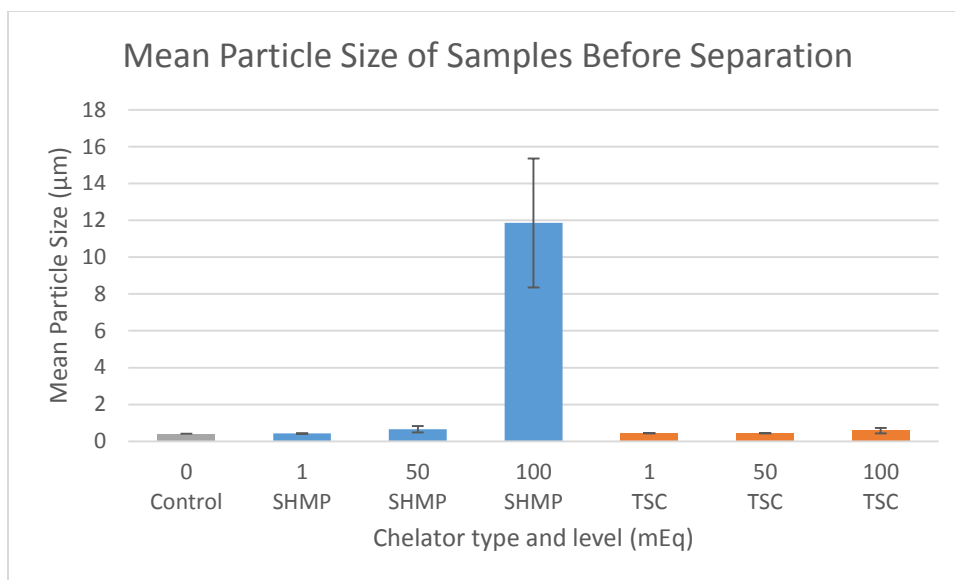


Figure 5.2. Means ( $\pm$ SE) showing the mean particle size ( $\mu\text{m}$ ) of the stock (unseparated) chelated samples. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate

### 5.1.3 pH

Once again, One-Way ANOVA was run with pH as the response and chelator/level combinations (at 7 levels) as the factor. These results indicate a statistically significant difference in mean pH based on chelator type/level combinations when compared with a control ( $p < 0.001$ ). Samples chelated with TSC showed an increasing pH as the chelator level was increased. Samples treated with SHMP showed that as chelator levels increased, pH initially increased and then decreased after large amounts (100 mEq) of chelator were added. These trends can be seen in Figure 5.3, below. Samples treated with 1 mEq of either chelator remained close to the pH of the control, with no statistically significant differences. The interesting peak in SHMP pH before decreasing was also observed by Mizuno and Lucey (2005) but could not fully be explained. It has been

suggested that this increase is due to the type of soluble calcium phosphate formed upon initial addition of SHMP and the exposed phosphoserine residues, similar to the pH increase seen upon formation of the soluble calcium citrate when TSC is added. The pH is thought to then decrease as the SHMP crosslinks the residues (Mizuno and Lucey, 2007).

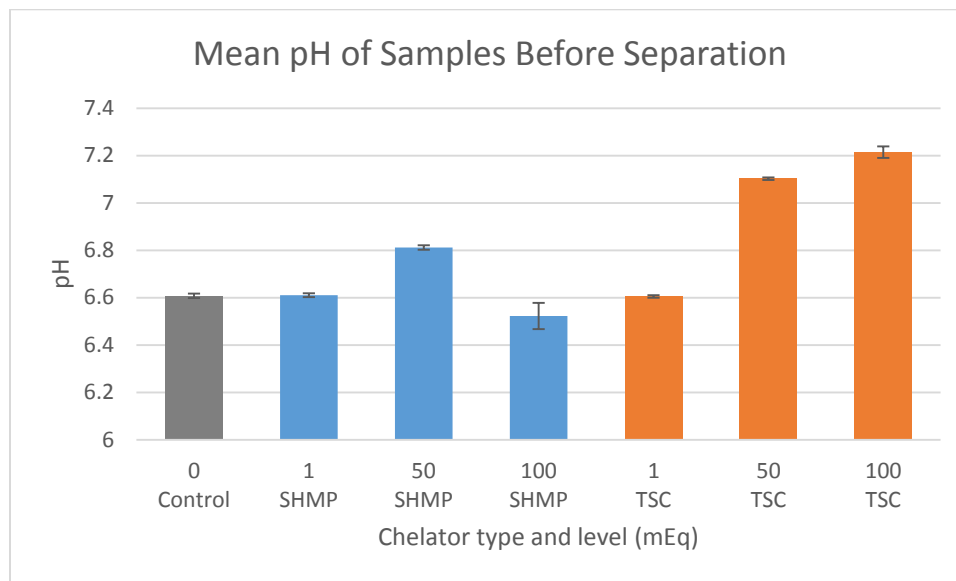


Figure 5.3. Means ( $\pm$ SE) showing the pH of the stock (unseparated) chelated samples. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate

## 5.2 Centrifugation

### 5.2.1 Turbidity

In the studies on the impact of ultracentrifugation, there is some evidence that the turbidity is impacted by centrifugal force ( $p = 0.026$ ) and by chelator level ( $p = 0.043$ ). The interactions between speed, level, and chelator were not found to be significant, nor was the effect of chelator type. The lack of significant difference in the type of chelator

used is important in a practical or industrial sense, as it allows wider freedom in the chelator selection process. This might allow the most cost effective or otherwise more desirable chelator to be used to chelate the product, as long as the optimum centrifugal force or optimum chelator level are used.

As the chelator level increased, there was notable decrease in the turbidity of the supernatant after centrifugation, with a mean turbidity of 0.3976 in samples chelated at 1 mEq levels, and a turbidity of 0.2075 in samples chelated at a level of 100 mEq. Figure 5.4 shows the steady decrease in turbidity as larger amounts of chelator are added. This is expected, showing that as larger amounts of chelator are added, more calcium is chelated out of the micelle and the micellar structure is broken down, resulting in smaller particles and less light scattering ability (Odagiri and Nickerson, 1965; Mizuno and Lucey, 2005; de Kort, 2012).

An increase in centrifugal force applied to samples also resulted in a decrease in turbidity of the supernatant. This falls in line with previous research and knowledge of centrifugation. The larger, denser, casein micelles will remain in the pellet, creating a less turbid supernatant. As the centrifugal force is increased, smaller molecules are forced out of the supernatant and into the sedimented pellet. Therefore, the statistical difference in turbidity seen between centrifugal force levels (Figure 5.5) shows that at higher forces (90,000xg) the turbidity is significantly lower than at low forces (30,000xg), because at higher forces, the molecules left in the supernatant are smaller and more opaque.

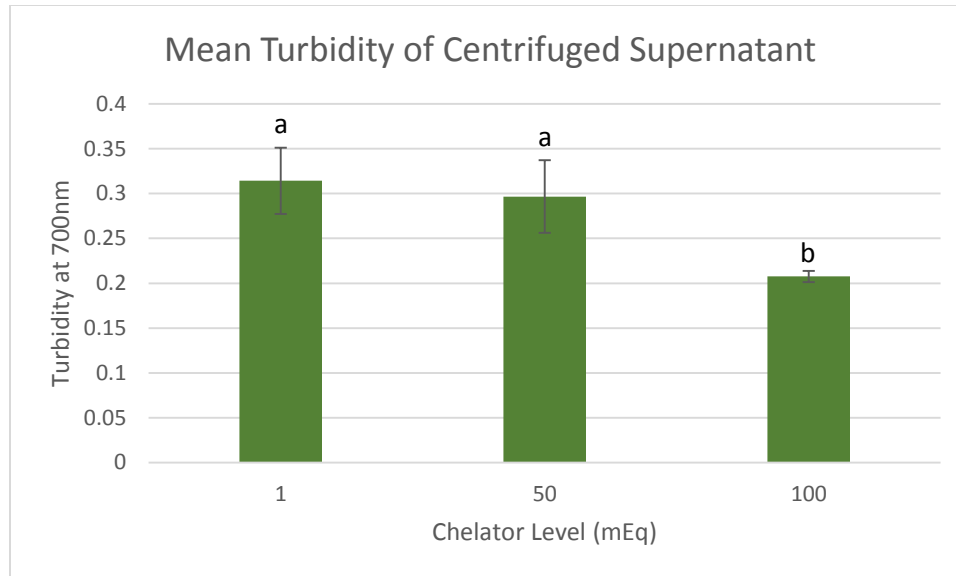


Figure 5.4. Means ( $\pm$ SE) showing the turbidity for the statistically significant effect of chelator level on the centrifuged supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ )

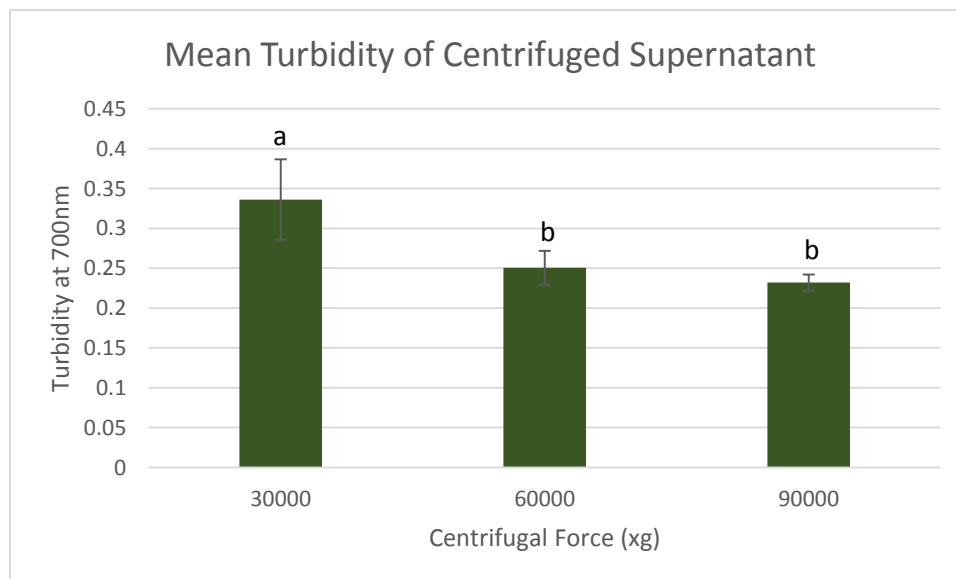


Figure 5.5. Means ( $\pm$ SE) showing the turbidity for the statistically significant effect of centrifugal force on the centrifuged supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ )

### 5.2.2 Protein Content

Caseins provide the majority of the protein content of milk, with 75-80% of the total protein being casein. The remaining 20% of milk protein, the whey proteins, are found in the milk serum and not sedimented by centrifugation. The presence of the whey proteins in the milk serum, or supernatant, can be seen in the control samples of the study, listed in Table 5.1. The protein content in these samples is thought to be contributed largely by whey proteins, since in an unchelated sample, all caseins are still in micellar form and found in the centrifuged pellet rather than the supernatant. As the casein micelle breaks down, the individual caseins are thought to be released from the larger micelle structure and may exist in the solution as smaller monomers, dimers, or small conglomerates of individual casein (de Kort et al., 2011). Therefore, as the micelle is disrupted and then centrifuged, there is an expectation that the amount of casein in the supernatant will increase. This is because the supernatant now contains not only the whey proteins but also the smaller less dense casein molecules that do not sediment out during centrifugation.

Protein content in the supernatant was analyzed, with mean protein levels ranging from  $0.41 \pm 0.06\%$  protein (1 mEq TSC, centrifuged at 90,000xg) to  $1.83 \pm 0.06\%$  protein 50 mEq SHMP, centrifuged at 30,000xg). The interaction between chelator type, chelator level, and centrifugal force was found to be associated with statistically significant differences in mean protein level in the sample supernatant ( $p < 0.001$ ). Because this interaction was statistically significant, the protein content was analyzed for each treatment combination and not for the main effects of chelator type, chelator level, or centrifugal force in isolation.

Table 5.1 shows the mean protein level of each of the treatment combinations. The samples that were treated with the lowest level (1 mEq) of both chelators show the lowest levels of protein in the supernatant for all centrifugal forces tested. This may be due to the low amount of micelle dissociation when treated with such a low level of chelator. The large micelles will pellet when centrifuged at all centrifugal forces tested, so the low amounts of protein in the supernatant of these samples suggests that the micelles have not dissociated. The low levels of protein seen in the supernatant of the unchelated control samples suggests that this assumption is true – that the large micelles pellet out when the chelator does not pull the calcium out of the micelle and destroy the structure. The overall protein content of the untreated samples (control) or samples treated with low levels (1 mEq) of chelator is far lower than the protein content of the samples treated with higher levels of chelator and therefore higher levels of micellar dissociation and caseins in the serum.

It was found that the treatment combination of 50 mEq SHMP centrifuged at 30,000xg had significantly higher amounts of protein released into the supernatant. Further research may be beneficial in determining the cause of this elevated protein in the supernatant. It may be that the SHMP has started to create a gel matrix within the casein system, but the low centrifugal force is not enough to cause the low density conglomerates to sediment out, whereas higher levels of SHMP and higher forces cause pelleting of the gel matrix, even though the casein micelle has been dissociated.

There appears to be no trend indicating that increasing centrifugal force and chelator level causes a steady increase or decrease on the amount of protein released for SHMP combinations. All SHMP 1 mEq samples are seen to have a lower protein level

than other SHMP samples, but the 50 mEq and 100 mEq samples show no trend in either centrifugal force or chelator level impacting the amount of protein. The results of combinations using TSC suggest that higher usage levels of TSC at any centrifugal force caused a greater amount of protein to be released. The TSC 100 mEq samples at all three centrifugal forces have significantly higher protein levels than the TSC 50 mEq (all centrifugal force levels) samples, and the TSC 1 mEq (all centrifugal force levels) samples are significantly lower than both of these.

Table 5.1. Mean ( $\pm$  pooled SE) protein levels (%) for centrifuged supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ). Control included for observation only, not as part of balanced design with factorial treatment structure. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate

Chelator	Chelator Level (mEq)	Centrifugal Force (xg)		
		30,000	60,000	90,000
SHMP	1	0.68 $\pm$ 0.03 <sup>h</sup>	0.56 $\pm$ 0.03 <sup>hi</sup>	0.53 $\pm$ 0.03 <sup>ij</sup>
	50	1.83 $\pm$ 0.03 <sup>a</sup>	1.25 $\pm$ 0.03 <sup>ef</sup>	1.46 $\pm$ 0.03 <sup>cd</sup>
	100	1.49 $\pm$ 0.03 <sup>bcd</sup>	1.54 $\pm$ 0.03 <sup>bc</sup>	1.35 $\pm$ 0.03 <sup>de</sup>
TSC	1	0.52 $\pm$ 0.03 <sup>ij</sup>	0.47 $\pm$ 0.03 <sup>ij</sup>	0.41 $\pm$ 0.03 <sup>j</sup>
	50	1.21 $\pm$ 0.03 <sup>f</sup>	1.18 $\pm$ 0.03 <sup>fg</sup>	1.07 $\pm$ 0.03 <sup>g</sup>
	100	1.61 $\pm$ 0.03 <sup>b</sup>	1.51 $\pm$ 0.03 <sup>bc</sup>	1.41 $\pm$ 0.03 <sup>cd</sup>
Control (no chelator)		0.59 $\pm$ 0.03	0.48 $\pm$ 0.03	0.44 $\pm$ 0.03

### 5.2.3 Alpha Casein

At a 0.05 significance level, the three way interaction between chelator type, chelator level, and centrifugal force is somewhat significant ( $p=0.048$ ) in determining the proportion of  $\alpha$  casein in the supernatant, but was not used for comparisons and further analysis. The method of quantifying data off of the SDS-PAGE gels is not highly accurate, and the sample size is small, so the decision was made to not analyze the interaction with a  $p$  value so close to the chosen significance level ( $\alpha = 0.05$ ). The two way interaction between chelator type and chelator level ( $p=0.051$ ) or interaction between chelator type and centrifugal force ( $p=0.143$ ) are also not significant. The two way interaction between chelator level and centrifugal force, however, is statistically significant ( $p = 0.005$ ) and shows significant differences between the mean  $\alpha$  casein proportion in samples chelated with 1 mEq of either chelator and those chelated with higher levels. At higher levels of chelator (50 mEq and 100 mEq) there is no significant difference between samples regardless of level of chelator or of centrifugal force, with mean  $\alpha$  casein amounts from 46.0% to 49.5%. Milk on average contains 50%  $\alpha$  casein, so these numbers suggest that most to all of the  $\alpha$  casein was released from the micelle into the supernatant at chelation levels higher than 50 mEq and at all centrifugal forces, even those as low as 30,000xg. In comparison, untreated control milk samples showed an average of 26.7% alpha casein when centrifuged at high levels, much lower than the proportions of alpha casein found in the chelated samples.

The samples chelated with 1 mEq of either chelator were significantly lower in the proportion of  $\alpha$  casein in the supernatant (Figure 5.6). This follows the findings in the protein analysis and turbidity, that at the low levels of chelator, there is very little casein



released from the micelle. This also expands upon that and suggests, as was reported by Horne (1998) and (Gaucheron, 2005) that the  $\alpha$  casein more tightly binds to the colloidal calcium phosphate because it has more phosphate groups than the other caseins. When a low amount of chelator (1 mEq) is added, the other caseins are readily released from the micelle, while  $\alpha$  casein remains tightly bound to the remaining CCP. There are significantly higher proportions of  $\alpha$  casein released from the 1 mEq treated samples at 30,000xg than at 90,000xg. This may be due to the inability of the low levels of centrifugal force to sediment out every casein micelle, whereas at higher centrifugal forces, the micelles more easily and more quickly are forced out of solution. This again is in line with the findings on turbidity and centrifugal force mentioned in section 5.2.1.

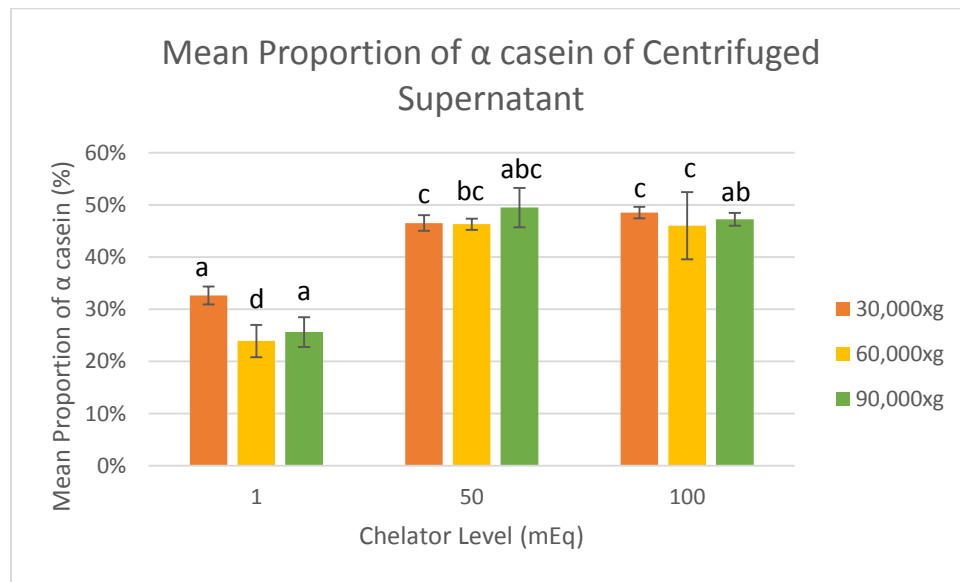


Figure 5.6. Means ( $\pm$ SE) showing the proportion of  $\alpha$  casein for the statistically significant interaction of chelator level and centrifugal force on the centrifuged supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ )

#### 5.2.4 Beta Casein

The amount of  $\beta$  casein is also found not be statistically significantly impacted by the three way interaction of chelator type, chelator level, and centrifugal force ( $p=0.108$ ), nor is it impacted by the two way interactions of chelator type and chelator level ( $p=0.342$ ) or chelator type and centrifugal force ( $p=0.359$ ). As seen with  $\alpha$  casein, the two way interaction between chelator level and centrifugal force is the only significant interaction when looking at the amount of  $\beta$  casein in sample supernatant ( $p<0.001$ ). The amount of  $\beta$  casein in the supernatant is inversely related to the amount of  $\alpha$  casein. It was addressed in section 5.2.3 that there was significantly lower proportions of  $\alpha$  casein found in the supernatant when the samples were treated with lower levels of chelator (1 mEq). The opposite is seen with  $\beta$  casein, with the largest mean percentages of  $\beta$  casein in the supernatant of samples that have been chelated with 1 mEq of either chelator (Figure 5.7). Because the casein content was analyzed as a total proportion, it cannot be determined if the amount of  $\alpha$  casein in the supernatant of some samples is causing a lower proportion of  $\beta$  casein, or vice versa, or even some combination of the two.

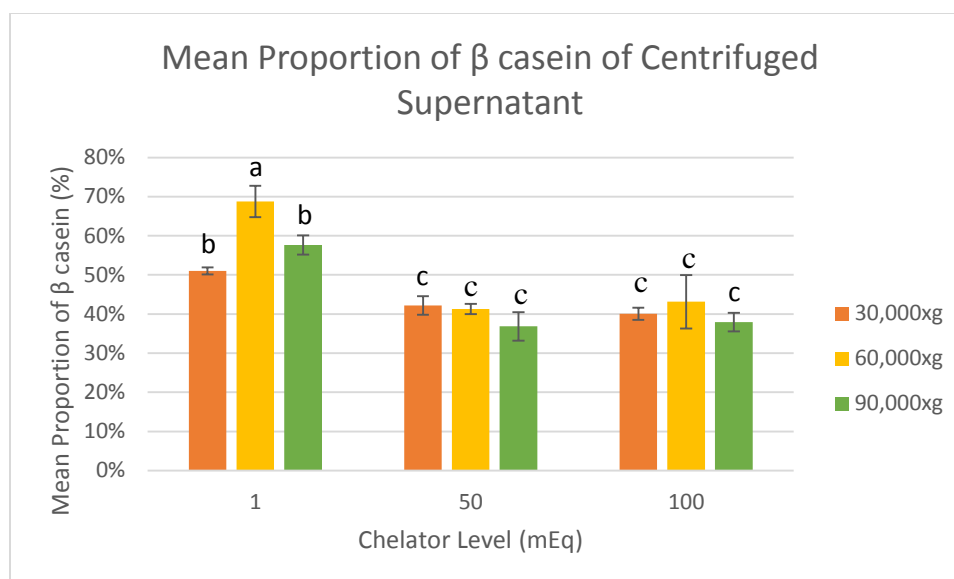


Figure 5.7. Means ( $\pm$ SE) showing the proportion of  $\beta$  casein for the statistically significant interaction of chelator level and centrifugal force on the centrifuged supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ )

### 5.2.5 Kappa Casein

The interaction between chelator type, chelator level, and centrifugal force is found to cause statistically significant differences in  $\kappa$  casein proportion in the sample supernatant ( $p=0.004$ ). The mean proportion of  $\kappa$  casein in sample supernatant ranged from  $6.50 \pm 0.01\%$  (1 mEq TSC, centrifuged at 60,000xg) and  $18.42 \pm 0.01\%$  (1 mEq TSC, centrifuged at 90,000xg). The mean  $\kappa$  casein proportion values are listed in Table 5.2. Though the Tukey comparison shows that there are statistically significant differences, there are no noticeable trends in the amount of  $\kappa$  casein in samples. Milk contains on average 10%  $\kappa$  casein, but has been recorded by some to have values anywhere from 6% to 15% (Fox and Brodorb, 2008; Farrell et al., 2004). Control samples ranged from  $6.91 \pm 0.36\%$  to  $13.57 \pm 0.36\%$   $\kappa$  casein. Because of this wide

range of accepted values and control values, as well as the lack of any noticeable trend within the data, it is difficult to propose that the proportion of  $\kappa$  casein is highly effected by this treatment combination.  $\kappa$  casein is also the least likely of the caseins to bind with the CCP because of its lack of a phosphoserine cluster, therefore, it is not surprising that the chelation of CCP from the micelle has the least significant effect on the proportion of  $\kappa$  casein in the supernatant (Dalglish, 1998).

Table 5.2.  $\kappa$  casein proportion (%  $\pm$  pooled SE) of centrifuged supernatant.

Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ). Control included for observation only, not as part of balanced design with factorial treatment structure. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate

Chelator	Chelator Level (mEq)	Centrifugal Force		
		30,000xg	60,000xg	90,000xg
SHMP	1	17.99 $\pm$ 0.01 <sup>ab</sup>	8.24 $\pm$ 0.01 <sup>ef</sup>	15.06 $\pm$ 0.01 <sup>abcd</sup>
	50	10.83 $\pm$ 0.01 <sup>edf</sup>	12.93 $\pm$ 0.01 <sup>bcde</sup>	13.54 $\pm$ 0.01 <sup>abcde</sup>
	100	11.26 $\pm$ 0.01 <sup>def</sup>	11.94 $\pm$ 0.01 <sup>cde</sup>	17.17 $\pm$ 0.01 <sup>abc</sup>
TSC	1	14.66 $\pm$ 0.01 <sup>abcd</sup>	6.50 $\pm$ 0.01 <sup>f</sup>	18.42 $\pm$ 0.01 <sup>a</sup>
	50	11.78 $\pm$ 0.01 <sup>def</sup>	11.98 $\pm$ 0.01 <sup>cde</sup>	13.84 $\pm$ 0.01 <sup>abcd</sup>
	100	11.63 $\pm$ 0.01 <sup>def</sup>	9.80 $\pm$ 0.01 <sup>def</sup>	12.48 $\pm$ 0.01 <sup>cde</sup>
Control (no chelator)		10.96 $\pm$ 0.01	6.91 $\pm$ 0.01	13.57 $\pm$ 0.01

### 5.2.6 Particle Size Analysis

The minimum detection level of the particle size analyzer used is 0.02 $\mu$ m and required an obscuration of at least 10% upon sample loading (Beckman Coulter, Brea, CA). None of the supernatant samples analyzed in this experiment were able to be analyzed for particle size because none, including unchelated control, were able to meet the obscuration minimum or be detected by the machine. This is supported by the previous turbidity and protein content findings that suggest that the addition of chelators accompanied by the use of centrifugal force to separate creates a supernatant void of casein micelles. The individual casein molecules, if present in the chelated supernatant, are likely too small to be analyzed by this device.

## 5.3 Temperature

After heat treatment was applied to samples, there was no formation of coagulum. The temperature of 70°C was selected because of its similarity to the pasteurization temperature of milk, and 90°C was selected as higher extreme. Literature suggests that the addition of chelators to milk causes greater heat stability (de Kort et al., 2012). This was true in this experiment also, as no coagulum at lower temperatures was found in the chelated samples. Because no coagulum was found, this experimental work was discontinued at this point.

## 5.4 Membrane Filtration

### 5.4.1 Turbidity

There were no statistically significant differences found in sample turbidity reading attributable to the interaction between chelator type and chelator level ( $p=0.166$ )

or to the main effects of chelator type ( $p = 0.324$ ) or chelator level ( $p=0.933$ ). The samples were very clear and turbidity readings at 700nm were all extremely low. This is expected, as the only things going through the membrane are likely water, lactose, minerals, which would produce a less turbid sample. Table 5.3 below lists the turbidity readings for the permeate of all treatment combinations. Because turbidity and protein content (Section 5.4.2) were both so low, it is thought that the membrane size used was too small for successful separation of any individual caseins from micelles or caseins combined in a gel matrix. The slightly higher turbidity readings and protein content in TSC treated with 100 mEq suggests that at high levels of chelation with a citrate, which does not create a gel matrix, there is possibility of separation of individual caseins if a slightly larger membrane size were used.

Table 5.3. Mean turbidity at 700nm ( $\pm$  pooled SE) for membrane filtered permeate. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ). Control included for observation only, not as part of balanced design with factorial treatment structure. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate

Chelator	Chelator Level (mEq)	Turbidity
SHMP	1	0.042 $\pm$ 0.01 <sup>a</sup>
	50	0.036 $\pm$ 0.01 <sup>a</sup>
	100	0.033 $\pm$ 0.01 <sup>a</sup>
TSC	1	0.036 $\pm$ 0.01 <sup>a</sup>
	50	0.041 $\pm$ 0.01 <sup>a</sup>
	100	0.048 $\pm$ 0.01 <sup>a</sup>
Control (no chelator)		0.032 $\pm$ 0.01

#### 5.4.2 Protein Content

The interaction between chelator type and chelator level was found to cause statistically significant differences in protein level in the sample permeate ( $p=0.006$ ). Mean protein amounts were extremely low in all samples, ranging from  $0.02 \pm 0.005\%$  to  $0.06 \pm 0.005\%$ , as shown in Table 5.4 below. The mean protein level of the control sample was  $0.03 \pm 0.005\%$ , which was not statistically different from any of the samples, except the sample treated with 100 mEq of TSC. 100 mEq of TSC had a mean protein level of  $0.06 \pm 0.005\%$ . While this is still a small amount of protein, it is significantly

more than the other samples. This may be because at such high levels of TSC, the casein micelle is fully dissociated, and unlike SHMP, TSC does not create a gel matrix within the proteins. Therefore, there is a higher chance of individual caseins making it through the membrane and into the permeate. Large significance should not be attached to these protein levels however, as it is a very small amount of protein, and the analysis of these samples was done for total nitrogen. This leaves the possibility that the nitrogen contributing to this increase is not true protein, but non protein nitrogen. Further nitrogen analysis could be done on these samples to determine if this small increase in the 100 mEq TSC sample is coming from true protein, or if it is in fact only non protein nitrogen.

Table 5.4. Mean protein levels ( $\% \pm$  pooled SE) for membrane filtered permeate. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ). Control included for observation only, not as part of balanced design with factorial treatment structure. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate

Chelator	Chelator Level (mEq)	Protein %
SHMP	1	$0.02 \pm 0.005^b$
	50	$0.02 \pm 0.005^b$
	100	$0.02 \pm 0.005^b$
TSC	1	$0.03 \pm 0.005^b$
	50	$0.03 \pm 0.005^b$
	100	$0.06 \pm 0.005^a$
Control (no chelator)		$0.03 \pm 0.005$



#### 5.4.3 Casein Content

SDS-PAGE analysis showed no visible bands in the region where casein proteins should be present. This suggests that there is no  $\alpha_s$ ,  $\beta$  or  $\kappa$  casein present in the permeate (Figure 5.8). This is confirmed by the analysis of the retentate, which shows strong  $\alpha_s$ ,  $\beta$  or  $\kappa$  casein bands (Figure 5.9). Because there was no reportable data from the permeate gel analysis, statistical analysis was not run on these results.

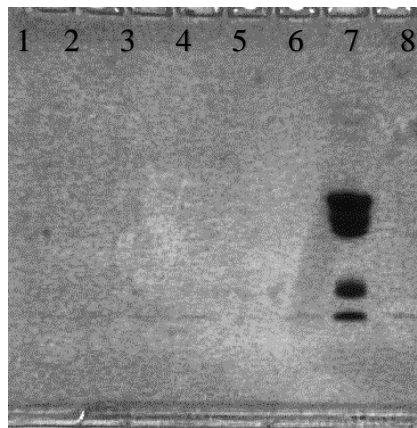


Figure 5.8. Denatured and reduced permeate samples run on a 12% SDS-PAGE gel and visualized through Coomassie staining Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate *Lane 1:* Control *Lane 2:* TSC 100 mEq *Lane 3:* SHMP 100mEq *Lane 4:* SHMP 1 mEq *Lane 5:* TSC 50 mEq *Lane 6:* SHMP 50 mEq *Lane 7:* Unfiltered milk *Lane 8:* TSC 1 mEq

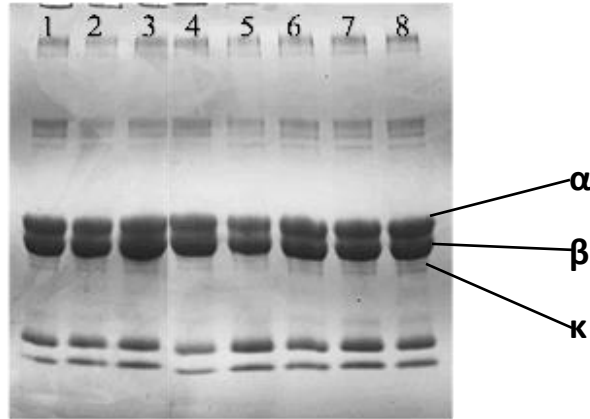


Figure 5.9. Denatured and reduced retentate samples run on a 12% SDS-PAGE gel and visualized through Coomassie staining Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate *Lane 1*: TSC 1 mEq *Lane 2*: TSC 100 mEq *Lane 3*: Unfiltered milk *Lane 4*: SHMP 50 mEq *Lane 5*: SHMP 1 mEq *Lane 6*: Control *Lane 7*: TSC 50 mEq *Lane 8*: SHMP 100 mEq

#### 5.4.4 Particle Size Analysis

The minimum detection level of the particle size analyzer used is 0.02 $\mu$ m and required an obscuration of at least 10% upon sample loading (Beckman Coulter, Brea, CA). None of the supernatant samples analyzed in this experiment were able to be analyzed for particle size because none were able to meet the obscuration minimum or be detected by the machine. This is supported by the previous turbidity and protein content findings that suggest that the addition of chelators and the use of centrifugal force to separate creates a supernatant void of casein micelles. The individual casein molecules present in the supernatant are likely too small to be analyzed by this device.

## 5.5 Acidification

### 5.5.1 Turbidity

The control samples saw a decrease in turbidity as the pH decreased, which is reasonable as the caseins coagulating would cause more to pellet out and less micelles in the supernatant. The interaction between chelator type, chelator level, and pH is statistically significant ( $p < 0.001$ ) in explaining differences in turbidity readings.

However, there was no consistent trend within the treated samples, especially as the amount of chelator was increased, seen in Table 5.5. In the samples treated with 50 mEq and 100 mEq SHMP, there is no statistical trend, and 50 mEq TSC shows higher turbidity in the samples treated with the middle two pHs. Only the samples treated with 1 mEq of either chelator show results similar to the control, and indicating that the caseins are coagulating more at lower pHs and creating a more casein poor supernatant. These results suggest that the addition of a chelator at high levels may cause an increased resistance to pH induced coagulation, and larger amounts of casein left in the supernatant at lower pH. This observation is confirmed with total protein analysis in section 5.5.2.

Table 5.5. Turbidity ( $\pm$  pooled SE) at 700nm for pH adjusted supernatant.

Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ). Control included for observation only, not as part of balanced design with factorial treatment structure. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate

Chelator Type	Chelator Level (mEq)	Adjusted pH			
		4.94	5.13	5.37	5.61
SHMP	1	$0.07 \pm 0.37^d$	$2.28 \pm 0.37^{ab}$	$2.53 \pm 0.37^{ab}$	$2.59 \pm 0.37^{ab}$
	50	$0.74 \pm 0.37^{cd}$	$0.25 \pm 0.37^{cd}$	$0.41 \pm 0.37^{cd}$	$0.16 \pm 0.37^d$
	100	$0.38 \pm 0.37^{cd}$	$0.19 \pm 0.37^d$	$0.20 \pm 0.37^{cd}$	$0.15 \pm 0.37^d$
TSC	1	$0.37 \pm 0.37^{cd}$	$2.19 \pm 0.37^{ab}$	$2.63 \pm 0.37^{ab}$	$2.59 \pm 0.37^{ab}$
	50	$0.64 \pm 0.37^{cd}$	$2.70 \pm 0.37^a$	$2.25 \pm 0.37^{ab}$	$0.90 \pm 0.37^c$
	100	$2.15 \pm 0.37^{ab}$	$1.99 \pm 0.37^b$	$0.67 \pm 0.37^{cd}$	$0.29 \pm 0.37^{cd}$
Control (no chelator)	0	$1.24 \pm 0.37$	$1.61 \pm 0.37$	$2.68 \pm 0.37$	$2.77 \pm 0.37$

### 5.5.2 Protein Content

The interaction between chelator type, chelator level, and sample pH is found to cause statistically significant differences in protein level in the sample supernatant ( $p<0.001$ ). Mean protein levels ranged from  $0.60 \pm 0.23\%$  to  $3.09 \pm 0.23\%$ . Noticeably, the amount of protein tends to increase as the pH increases. This is reasonable, as it is widely known that decreasing pH will cause coagulation of the proteins. This trend is

statistically significant for the samples treated with 1 mEq SHMP. There is significantly more protein in the higher pH samples than the lower pH samples. This trend, as well as all mean protein levels, can be found in Table 5.6. The presence of the whey proteins in the milk serum, or supernatant, can be seen in the control samples of the study, also listed in Table 5.6. The protein content in these samples is thought to be contributed largely by whey proteins, since in an unchelated sample, all caseins are assumed to be still in micellar form and found in the coagulated pellet rather than the supernatant. It is also noticeable that the mean protein level of samples remains higher at lower pHs in the samples treated with higher levels of chelator, likely because the caseins are no longer in micellar form at this point, and less susceptible to pH based coagulation.

Table 5.6. Mean protein levels (%  $\pm$  pooled SE) for pH adjusted supernatant.

Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ ). Control included for observation only, not as part of balanced design with factorial treatment structure. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate

Chelator	Chelator Level (mEq)	Adjusted pH			
		4.94	5.13	5.37	5.61
SHMP	1	0.60 $\pm$ 0.23 <sup>j</sup>	1.08 $\pm$ 0.23 <sup>i</sup>	1.65 $\pm$ 0.23 <sup>h</sup>	2.42 $\pm$ 0.23 <sup>defg</sup>
	50	2.55 $\pm$ 0.23 <sup>i</sup>	2.85 $\pm$ 0.23 <sup>abcd</sup>	2.94 $\pm$ 0.23 <sup>abc</sup>	3.09 $\pm$ 0.23 <sup>a</sup>
	100	2.85 $\pm$ 0.23 <sup>abcd</sup>	2.69 $\pm$ 0.23 <sup>abcde</sup>	3.00 $\pm$ 0.23 <sup>ab</sup>	3.02 $\pm$ 0.23 <sup>a</sup>
TSC	1	0.65 $\pm$ 0.23 <sup>ij</sup>	0.72 $\pm$ 0.23 <sup>ij</sup>	2.40 $\pm$ 0.23 <sup>efg</sup>	2.74 $\pm$ 0.23 <sup>abcde</sup>
	50	1.08 $\pm$ 0.23 <sup>cdef</sup>	2.01 $\pm$ 0.23 <sup>gh</sup>	2.58 $\pm$ 0.23 <sup>bcdef</sup>	2.24 $\pm$ 0.23 <sup>fg</sup>
	100	1.99 $\pm$ 0.23 <sup>gh</sup>	2.14 $\pm$ 0.23 <sup>fg</sup>	2.84 $\pm$ 0.23 <sup>abcd</sup>	2.82 $\pm$ 0.23 <sup>abcde</sup>
Control (no chelator)	0	0.69 $\pm$ 0.23	1.31 $\pm$ 0.23	2.08 $\pm$ 0.23	2.40 $\pm$ 0.23

### 5.5.3 Alpha Casein

There were no statistically significant interactions or main effects for the proportion of  $\alpha$  casein in the pH adjusted sample supernatant. The mean  $\alpha$  casein ranges from  $44.79 \pm 3.90\%$  to  $55.76 \pm 3.90\%$ , an acceptable range for  $\alpha$  casein in milk.

The effect of acidification on the casein content of milk was studied by Law (1996), and it was found that acidification caused fluctuation in the levels of  $\beta$  casein and  $\kappa$  casein in supernatant, but that the  $\alpha$  casein levels remained relatively stable. These

results (as expanded upon in sections 5.5.4 and 5.5.5) are in line with those findings, and suggests that the use of a chelator does not have an effect on the types of casein released upon acidification of samples.

Two samples, 1 mEq SHMP pH 4.94, and 1 mEq TSC pH 4.94 showed no visible bands in the region where casein proteins should be present. This suggests that the casein content of these samples was still in micellar form, and that at the low pH of 4.94, all of the casein micelles were bound in a coagulated mass, leaving minimal amounts of protein in the supernatant. This is backed up by the control samples that also saw a lack of casein in the samples adjusted to low pH. Figure 5.10 shows the distinct lack of bands in the 1 mEq SHMP pH 4.94, and 1 mEq TSC pH 4.94 samples.

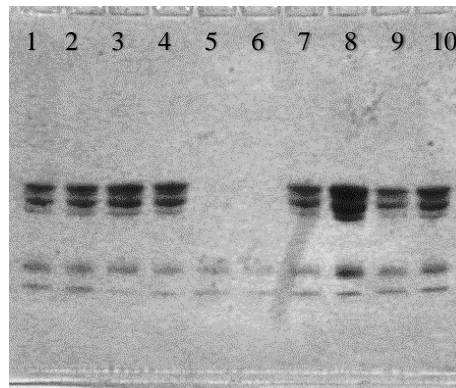


Figure 5.10. Denatured and reduced supernatant samples run on a 12% SDS-PAGE gel and visualized through Coomassie staining. Control = No chelator added, SHMP = Sodium Hexametaphosphate, TSC = Trisodium Citrate *Lane 1:* TSC 1 mEq, pH 5.14 *Lane 2:* TSC 100 mEq, pH 5.14 *Lane 3:* TSC 100mEq 5.37 *Lane 4:* SHMP 100Meq, pH 5.61 *Lane 5:* TSC 1 mEq pH 4.94 *Lane 6:* SHMP 1 mEq pH 4.94 *Lane 7:* Control, pH 4.94 *Lane 8:* TSC 1 mEq pH 5.37 *Lane 9:* SHMP 50 mEq pH 4.94 *Lane 10:* TSC 50 mEq pH 5.14

#### 5.5.4 Beta Casein

The sample pH has a somewhat significant effect on the proportion of  $\beta$  casein in supernatant ( $p = 0.010$ ). The main effects plot (Figure 5.11) shows the interesting trend in the proportion of  $\beta$  casein, a fluctuation that consists of first a large decrease in  $\beta$  casein proportionally to a mean of 37.00%, and then a spike, returning closer to the 40%  $\beta$  casein (40.76%) that is commonly found in untreated milk, as the pH is lowered. The mean proportion of  $\beta$  casein ranges from  $37.00 \pm 1.32\%$  to  $40.76 \pm 2.66\%$ . Tukey comparisons show only a statistically significant difference between a pH of 5.37 and 4.94. It is important to note once again, that casein content was measured as a total proportion, so the decrease in  $\beta$  casein proportionally at pH 5.37 may also be tied to the inverse, proportional increase of  $\kappa$  casein at this same pH, noted in section 5.5.5.

As was mentioned in section 5.5.3, two samples, 1 mEq SHMP pH 4.94, and 1 mEq TSC pH 4.94 showed no visible bands in the region where casein proteins should be present. Therefore, no data could be reported for those treatment combinations.



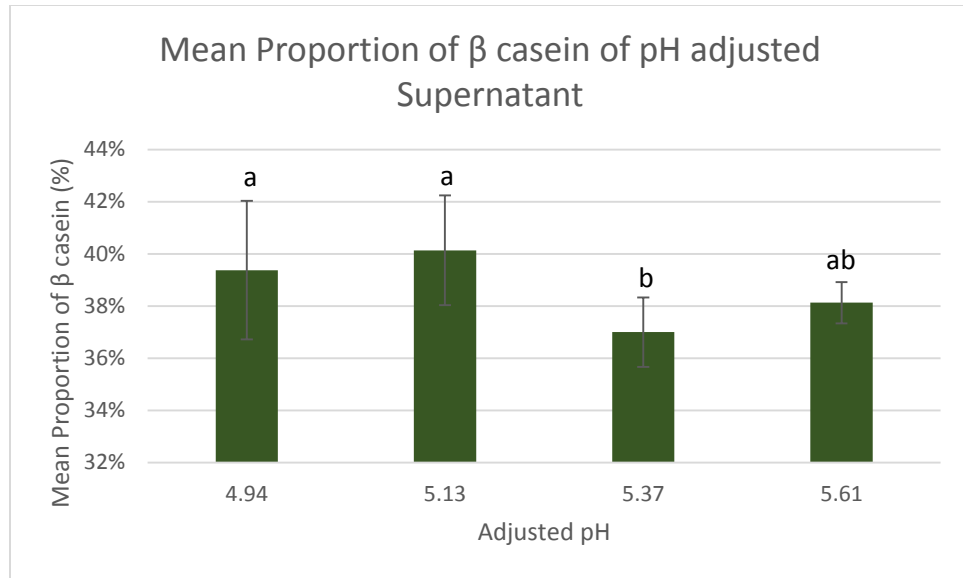


Figure 5.11. Means ( $\pm$ SE) for the statistically significant effect of adjusted pH on mean proportion of  $\beta$  casein in sample supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ )

#### 5.5.5 Kappa Casein

The amount of  $\kappa$  casein is also found not be statistically significantly impacted by the two way interactions of chelator type and chelator level ( $p=0.190$ ), chelator type and adjusted pH ( $p=0.265$ ) or by the main effect of chelator type ( $p=0.811$ ). Chelator level ( $p = 0.007$ ) and sample adjusted pH ( $p = 0.002$ ) were both found to significantly impact the proportion of  $\kappa$  casein in sample supernatant. Figure 5.12, below, shows the effect of chelator level and pH (Figure 5.13) on proportion of  $\kappa$  casein.

As the amount of chelator added to the system increases, the amount of  $\kappa$  casein in the supernatant appears to increase. The increasing chelator concentration should disrupt the micelle and cause more of the individual caseins to be present in the supernatant. This suggests that  $\kappa$  casein may be more sensitive to the disruption of micelles and may be more readily removed from the micelle. At low levels (1 mEq) of

chelator,  $9.72 \pm 1.57\%$  of the casein in supernatant is  $\kappa$  casein. This increases to  $12.53 \pm 0.85\%$   $\kappa$  casein when 100 mEq are added.

The effect of pH on proportion of  $\kappa$  casein is notably the inverse of the effect of pH on  $\beta$  casein levels. Although this spike in  $\kappa$  casein at pH 5.37 (and similar decrease in  $\beta$  casein) appears interesting, it is not a clear enough difference to gather much attention. Statistically, there is no difference in the proportion of  $\kappa$  casein between pH of 4.94, 5.37, and 5.61, with only a statistical difference between pH 5.13 and pH's of 5.37 and 5.61. These differences are noted in Figure 5.9 for  $\beta$  casein and Figure 5.11 for  $\kappa$  casein.

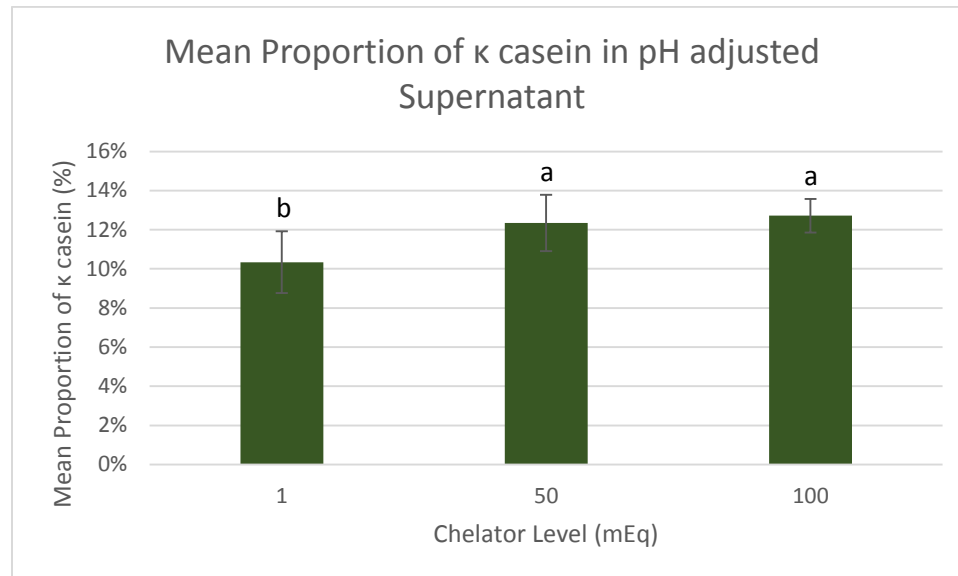


Figure 5.12. Means ( $\pm$ SE) showing the mean proportion of  $\kappa$  casein in sample supernatant for the statistically significant effect of chelator level on the pH adjusted sample supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ )

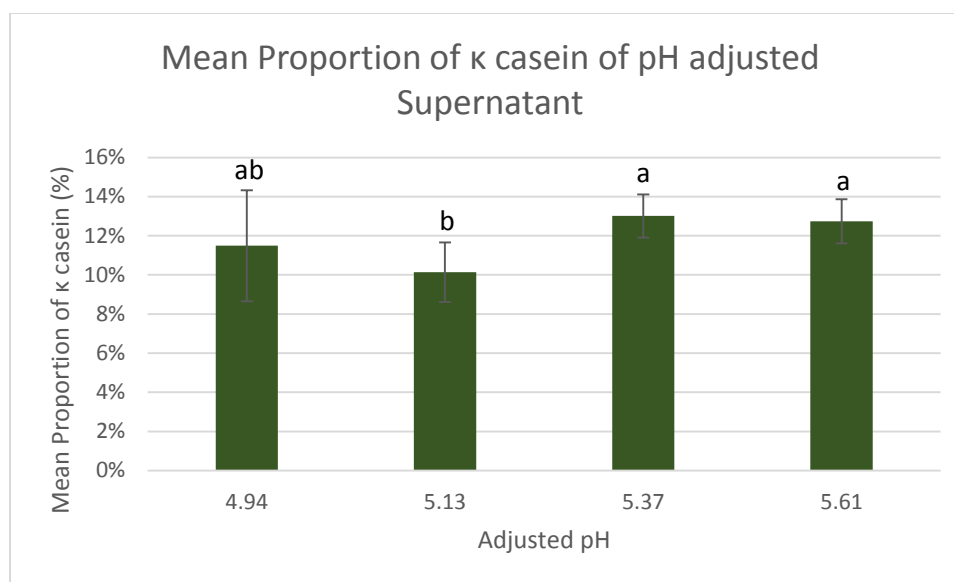


Figure 5.13. Means ( $\pm$ SE) showing the mean proportion of  $\kappa$  casein in sample supernatant for the statistically significant effect of pH on the pH adjusted sample supernatant. Samples that do not share a letter are significantly different by Tukey comparison ( $\alpha=0.05$ )

#### 5.5.6 Particle Size Analysis

The interaction between chelator type, chelator level, and sample pH is found to cause statistically significant differences in particle size in the sample supernatant ( $p<0.001$ ). Mean particle size ranged from  $0.25 \pm 4.88 \mu\text{m}$  (1 mEq SHMP, pH 5.61) to  $214.13 \pm 4.88 \mu\text{m}$  (100 mEq SHMP, pH 5.37), with all treatment combination mean particle sizes listed in Table 5.7. The sample treated with 100 mEq SHMP at pH 5.37 showed the largest average particle size, approximately 200  $\mu\text{m}$  larger than any of the other samples. This is an interesting point for further research, as it is possible that this chelator at such high levels creates a loose gel network, and as the pH is dropped, the large gel matrix first binds to create larger molecules, but this loose network is not yet dense enough to drop out of suspension and into the sedimented pellet, rather it remains

in the supernatant. When the pH is dropped lower, the matrix tightens and becomes dense enough to be pelleted. This could be advantageous in industrial work looking for increased viscosity and gel texture in liquid products with ability to stay in suspension. Further research would demonstrate if this is truly happening, Mizuno and Lucey (2007) and de Kort (2012) have studied the ability of polyphosphates to cross link the caseins, but an exact mechanism or understanding has not yet been established. There is also possibility that the high levels of SHMP completely broke apart the micelle, and that at pH 5.37 (the isoionic pH of  $\alpha_{s2}$  casein) the  $\alpha_{s2}$  caseins are released from the micelle. When the  $\alpha_{s2}$  and  $\kappa$  casein are the only released from the micelle, the hydrophilic nature of these caseins allows them to bind both with each other and with the water molecules, creating a larger overall matrix. This particle size then decreases as the pH is decreased, when the  $\alpha_{s1}$  and  $\beta$  caseins are available in the supernatant also and binding with the  $\alpha_{s2}$  instead of allowing the larger molecules bound with water. The large particles size in control samples as the pH is lowered, however, suggests that there are remaining coagulated proteins in the supernatant. Centrifuging samples for a longer time and at higher centrifugal forces may be recommended to ensure that all coagulated proteins are removed from supernatant.

Table 5.7. Mean particle size ( $\mu\text{m} \pm$  pooled SE) for pH adjusted supernatant.

Samples that do not share a letter are significantly different by Tukey comparison

( $\alpha=0.05$ ). Control included for observation only, not as part of balanced design

with factorial treatment structure. Control = No chelator added, SHMP = Sodium

Hexametaphosphate, TSC = Trisodium Citrate

Chelator	Chelator Level (mEq)	Adjusted pH			
		4.94	5.13	5.37	5.61
SHMP	1	$0.75 \pm 4.88^{\text{de}}$	$4.70 \pm 4.88^{\text{de}}$	$0.51 \pm 4.88^{\text{e}}$	$0.25 \pm 4.88^{\text{e}}$
	50	$9.892 \pm 4.88^{\text{bcd}}$	$15.33 \pm 4.88^{\text{bc}}$	$6.63 \pm 4.88^{\text{cde}}$	$7.87 \pm 4.88^{\text{cde}}$
	100	$17.45 \pm 4.88^{\text{b}}$	$0.51 \pm 4.88^{\text{e}}$	$214.13 \pm 4.88^{\text{a}}$	$6.45 \pm 4.88^{\text{cde}}$
TSC	1	$0.70 \pm 4.88^{\text{de}}$	$0.54 \pm 4.88^{\text{e}}$	$0.61 \pm 4.88^{\text{e}}$	$0.55 \pm 4.88^{\text{e}}$
	50	$2.04 \pm 4.88^{\text{de}}$	$0.58 \pm 4.88^{\text{e}}$	$0.60 \pm 4.88^{\text{e}}$	$0.57 \pm 4.88^{\text{e}}$
	100	$0.72 \pm 4.88^{\text{de}}$	$0.49 \pm 4.88^{\text{e}}$	$0.51 \pm 4.88^{\text{e}}$	$0.54 \pm 4.88^{\text{e}}$
Control	0	$5.52 \pm 4.88$	$6.51 \pm 4.88$	$6.37 \pm 4.88$	$0.37 \pm 4.88$

## 6. CONCLUSIONS AND FURTHER RESEARCH

The main objectives of this research were to explore the use of calcium chelators to dissociate the casein micelle and to determine the feasibility of different separation methods commonly used in industry on the chelated micelle to create different casein fractions. Three original hypothesis were set forth. It was hypothesized that the type of chelator used and usage level would affect the amount of dissociation, that the partitioning of individual caseins from the disrupted micelle would be effected by chelator type and usage level within each separation method used, and that the partitioning of individual casein could be manipulated by variations within the separation method used.

The results of this study show that the usage of different chelators and chelator levels can affect the amount of dissociation of the casein micelle. It is seen that SHMP is a stronger overall chelator, with less chelator being needed to create a less turbid (more dissociated) product. As the usage levels of either chelator increased, the turbidity of samples decreased also, suggesting an increase in dissociation. The addition of 50 mEq of chelator demonstrates the most drastic change between the two chelators in turbidity readings, with SHMP showing a drastic drop in turbidity, thus suggesting a more dissociated solution at this point and TSC showing a more stepwise gradient, with greater dissociation not present until closer to 100 mEq addition.

There was less conclusive evidence that the partitioning of individual caseins was affected by the changes in chelator type and chelator level. In the centrifugation study, it was seen that there was an increase in the proportion of  $\alpha$  casein in samples with higher levels (50 mEq and 100 mEq) of either chelator, and a corresponding decrease in  $\beta$  casein

at these levels. There was no conclusive evidence of a trend in the proportion in  $\kappa$  casein in the supernatant of centrifuged samples. The samples separated by acidification showed no trend in partitioning of  $\alpha$  or  $\beta$  casein based on chelator type and usage level. The proportion of  $\kappa$  casein was effected by chelator level, with larger proportions of  $\kappa$  casein found in the samples treated with higher levels of chelator. This suggests that  $\kappa$  casein may be more sensitive to the disruption of micelles and more readily removed from the micelle.

It was found that varying the amount of centrifugal force used to separate or partition the caseins in the centrifugation study may have an effect on the partitioning of the individual caseins. Varying the pH of the sample used to partition the caseins in the acidification study was also found to have a slight impact on the partitioning. Usage of higher centrifugal force was tied to an increase in  $\alpha$  casein proportion in the supernatant of samples with high levels of chelator and a corresponding decrease in the  $\beta$  casein proportion of these samples. The decrease of pH to 5.37 shows an interesting spike in the  $\beta$  casein proportion, and a decrease in the  $\kappa$  casein proportion. This trend does not continue as the pH is decreased or increased, however.

This research provides a modest base for further work into the partitioning of individual caseins from chelated milk products. It is practical to further explore the use of membrane filtration as an option for separation using a larger membrane size, as it is seen that little to no protein was able to get through the small pore size and that SHMP may create a larger gel matrix within the sample, creating a need for an even larger membrane pore size. Other future direction for research includes further verification of the casein content of samples using a more precise analysis method, such as HPLC to determine

casein content on an individually quantifiable basis, rather than as an overall proportion.

These insights may provide value to the dairy industry not in a singular product or process, but rather opens many different options as a way to incorporate higher protein levels into products as well as the potential to vary functionalities of products.



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## APPENDICES

### Appendix A- Unseparated Sample Raw Data

Chelator	Level	Turbidity	pH	Particle Size (µm)
SHMP	1	2.451	6.61	0.427
SHMP	1	2.438	6.62	0.424
SHMP	1	2.372	6.61	0.421
SHMP	1	2.426	6.6	0.419
SHMP	50	0.15	6.81	0.589
SHMP	50	0.189	6.82	0.541
SHMP	50	0.189	6.82	0.915
SHMP	50	0.188	6.8	0.586
SHMP	100	0.111	6.47	10.26
SHMP	100	0.1	6.51	8.23
SHMP	100	0.097	6.51	16.4
SHMP	100	0.101	6.6	12.543
TSC	1	2.52	6.61	0.445
TSC	1	2.616	6.6	0.441
TSC	1	2.743	6.6	0.441
TSC	1	2.444	6.61	0.432
TSC	50	1.231	7.1	0.443
TSC	50	1.348	7.11	0.439
TSC	50	1.298	7.1	0.424
TSC	50	1.251	7.1	0.421
TSC	100	0.223	7.18	0.791
TSC	100	0.183	7.22	0.5
TSC	100	0.174	7.23	0.555
TSC	100	0.188	7.23	0.474
Control		2.79	6.62	0.419
Control		2.8	6.61	0.414
Control		2.76	6.6	0.417
Control		2.82	6.6	0.415

# Appendix B- Centrifugation Study Raw Data

Chelator	Level	Force	Protein	$\alpha$	$\beta$	$\kappa$	Turbidity
SHMP	1	30000	0.62	33.39%	49.85%	16.76%	0.469
SHMP	1	30000	0.65	30.71%	50.94%	18.35%	0.448
SHMP	1	30000	0.70	34.10%	48.82%	17.08%	0.4535
SHMP	1	30000	0.76	28.94%	51.28%	19.77%	0.598
SHMP	1	60000	0.54	27.36%	66.88%	5.77%	0.283
SHMP	1	60000	0.51	28.71%	61.71%	9.58%	0.271
SHMP	1	60000	0.57	28.72%	67.10%	4.18%	0.2775
SHMP	1	60000	0.60	26.53%	60.04%	13.44%	0.28
SHMP	1	90000	0.57	28.78%	56.98%	14.23%	0.265
SHMP	1	90000	0.48	29.09%	52.46%	18.44%	0.246
SHMP	1	90000	0.53	30.30%	56.66%	13.04%	0.249
SHMP	1	90000	0.54	27.17%	58.28%	14.55%	0.251
SHMP	50	30000	1.86	49.86%	39.72%	10.42%	0.564
SHMP	50	30000	1.87	45.19%	46.18%	8.63%	0.197
SHMP	50	30000	1.71	49.34%	36.55%	14.11%	0.4595
SHMP	50	30000	1.87	47.74%	42.09%	10.17%	0.466
SHMP	50	60000	1.26	45.34%	41.28%	13.38%	0.232
SHMP	50	60000	1.26	46.30%	38.54%	15.16%	0.188
SHMP	50	60000	1.25	44.72%	43.29%	11.99%	0.2165
SHMP	50	60000	1.23	48.40%	40.41%	11.19%	0.231
SHMP	50	90000	1.41	52.20%	36.03%	11.77%	0.225
SHMP	50	90000	1.41	56.52%	30.84%	12.64%	0.194
SHMP	50	90000	1.47	49.91%	35.04%	15.05%	0.206
SHMP	50	90000	1.55	51.66%	33.64%	14.70%	0.2
SHMP	100	30000	1.48	49.84%	40.25%	9.91%	0.187
SHMP	100	30000	1.50	48.30%	39.75%	11.95%	0.193
SHMP	100	30000	1.46	50.10%	40.35%	9.54%	0.1865
SHMP	100	30000	1.51	48.28%	38.06%	13.66%	0.186
SHMP	100	60000	1.51	42.31%	44.29%	13.40%	0.205
SHMP	100	60000	1.50	43.11%	45.79%	11.09%	0.181
SHMP	100	60000	1.60	42.61%	44.62%	12.78%	0.195
SHMP	100	60000	1.54	44.90%	44.62%	10.48%	0.188
SHMP	100	90000	1.37	45.72%	36.79%	17.49%	0.215
SHMP	100	90000	1.36	46.41%	35.22%	18.37%	0.202
SHMP	100	90000	1.33	50.69%	32.40%	16.91%	0.2155
SHMP	100	90000	1.35	48.41%	35.65%	15.94%	0.2155
TSC	1	30000	0.52	35.33%	51.97%	12.70%	0.366
TSC	1	30000	0.47	30.31%	52.78%	16.91%	0.313

Chelator	Level	Force	Protein	$\alpha$	$\beta$	$\kappa$	Turbidity
TSC	1	30000	0.60	35.58%	50.76%	13.66%	0.328
TSC	1	30000	0.51	32.73%	51.90%	15.37%	0.3355
TSC	1	60000	0.48	18.06%	74.22%	7.71%	0.283
TSC	1	60000	0.47	20.19%	73.57%	6.24%	0.269
TSC	1	60000	0.46	22.04%	73.84%	4.12%	0.274
TSC	1	60000	0.48	19.64%	72.43%	7.93%	0.267
TSC	1	90000	0.35	22.07%	57.21%	20.71%	0.285
TSC	1	90000	0.44	23.21%	55.40%	21.39%	0.241
TSC	1	90000	0.39	25.62%	59.88%	14.51%	0.2585
TSC	1	90000	0.48	18.58%	64.32%	17.10%	0.2315
TSC	50	30000	1.20	45.05%	46.80%	8.15%	0.34
TSC	50	30000	1.25	45.08%	40.79%	14.13%	0.37
TSC	50	30000	1.17	44.73%	43.93%	11.34%	0.365
TSC	50	30000	1.23	45.01%	41.48%	13.50%	0.332
TSC	50	60000	1.17	47.92%	38.92%	13.16%	0.337
TSC	50	60000	1.16	47.11%	41.61%	11.28%	0.329
TSC	50	60000	1.23	44.05%	43.63%	12.32%	0.3345
TSC	50	60000	1.17	46.28%	42.55%	11.17%	0.343
TSC	50	90000	1.02	53.30%	33.83%	12.87%	0.268
TSC	50	90000	1.03	48.74%	35.96%	15.29%	0.211
TSC	50	90000	1.19	41.47%	44.88%	13.65%	0.2455
TSC	50	90000	1.06	41.88%	44.58%	13.55%	0.266
TSC	100	30000	1.59	50.44%	36.88%	12.68%	0.228
TSC	100	30000	1.63	45.72%	43.09%	11.19%	0.223
TSC	100	30000	1.62	47.92%	39.01%	13.07%	0.2275
TSC	100	30000	1.61	47.45%	42.96%	9.59%	0.2305
TSC	100	60000	1.54	60.05%	30.62%	9.33%	0.219
TSC	100	60000	1.52	59.98%	27.58%	12.44%	0.198
TSC	100	60000	1.53	35.17%	56.35%	8.47%	0.2005
TSC	100	60000	1.45	39.82%	51.23%	8.96%	0.2105
TSC	100	90000	1.43	47.32%	41.77%	10.92%	0.221
TSC	100	90000	1.43	46.88%	40.15%	12.97%	0.216
TSC	100	90000	1.35	47.23%	41.27%	11.50%	0.217
TSC	100	90000	1.41	45.19%	40.27%	14.54%	0.22
Control		30000	0.54	39.47%	49.88%	10.65%	0.342
Control		30000	0.60	39.86%	47.09%	13.05%	0.317
Control		30000	0.58	36.91%	53.26%	9.83%	0.172
Control		30000	0.64	42.01%	47.67%	10.32%	0.2015
Control		60000	0.41	36.41%	56.37%	7.22%	0.303
Control		60000	0.49	32.21%	58.36%	9.43%	0.294

Chelator	Level	Force	Protein	$\alpha$	$\beta$	$\kappa$	Turbidity
Control		60000	0.54	27.88%	67.36%	4.76%	0.295
Control		60000	0.47	27.26%	66.51%	6.23%	0.2995
Control		90000	0.43	26.72%	57.77%	15.50%	0.301
Control		90000	0.44	35.49%	54.08%	10.43%	0.238
Control		90000	0.48	28.13%	56.87%	15.01%	0.3125
Control		90000	0.40	32.28%	54.37%	13.35%	0.2405

# Appendix C- Membrane Filtration Study Raw Data

Chelator	Level	Protein	Turbidity
SHMP	1	0.03	0.05
SHMP	1	0.03	0.041
SHMP	1	0.02	0.034
SHMP	1	0.02	0.043
SHMP	2	0.02	0.042
SHMP	2	0.03	0.032
SHMP	2	0.01	0.037
SHMP	2	0.01	0.031
SHMP	3	0.03	0.056
SHMP	3	0.04	0.043
SHMP	3	0.02	0.023
SHMP	3	0.01	0.01
TSC	1	0.03	0.033
TSC	1	0.04	0.032
TSC	1	0.02	0.033
TSC	1	0.03	0.045
TSC	2	0.03	0.046
TSC	2	0.03	0.034
TSC	2	0.02	0.036
TSC	2	0.02	0.046
TSC	3	0.05	0.045
TSC	3	0.05	0.042
TSC	3	0.07	0.056
TSC	3	0.07	0.044
Control		0.02	0.034
Control		0.03	0.042
Control		0.03	0.029
Control		0.02	0.411



# Appendix D- Acidification Study Raw Data

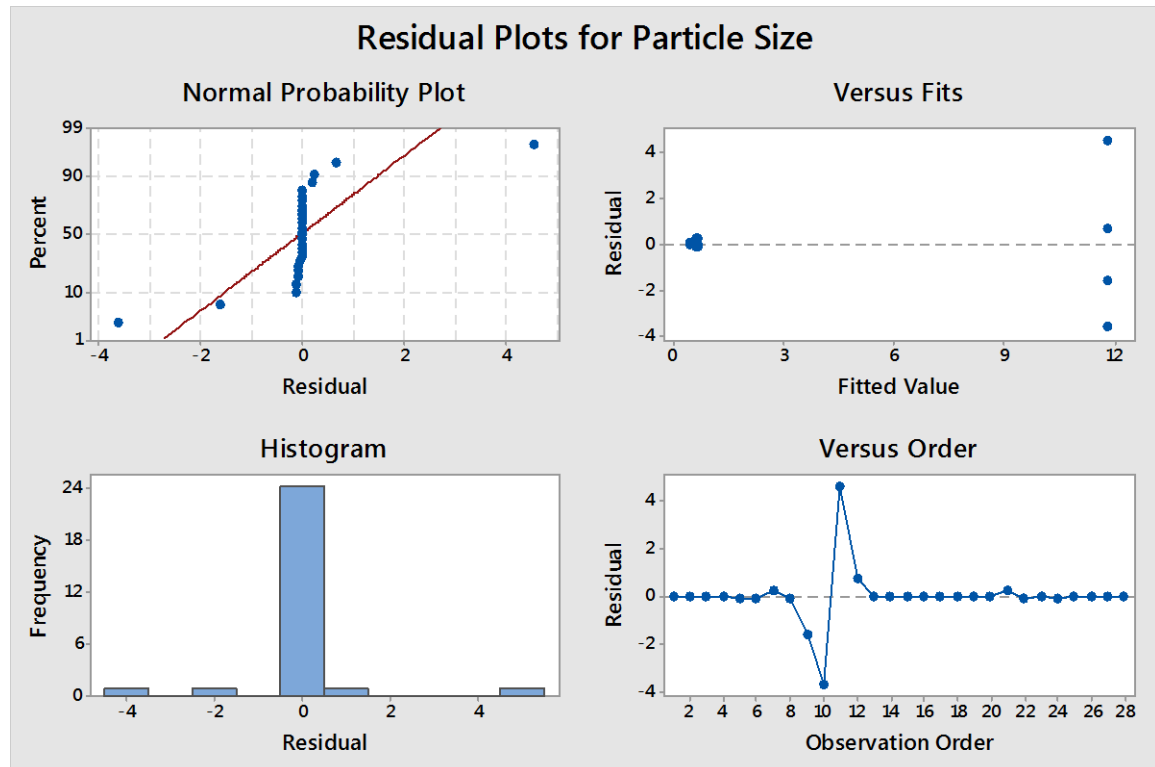
Chelator	Level	pH	Protein	$\alpha$ casein	$\beta$	$\kappa$	Turbidity	Particle Size
SHMP	1	4.94	0.61				0.0995	0.752
SHMP	1	4.94	0.63				0.0370	0.745
SHMP	1	4.94	0.58				0.0640	0.749
SHMP	1	4.94	0.59				0.0750	0.749
SHMP	1	5.13	1.16	0.46	0.44	0.10	2.3655	5.982
SHMP	1	5.13	1.00	0.48	0.49	0.03	1.8590	0.602
SHMP	1	5.13	1.11	0.46	0.43	0.11	2.6910	6.179
SHMP	1	5.13	1.05	0.39	0.53	0.07	2.2195	6.025
SHMP	1	5.37	1.44	0.51	0.43	0.05	2.6005	0.502
SHMP	1	5.37	1.71	0.52	0.39	0.09	2.4170	0.502
SHMP	1	5.37	1.93	0.56	0.31	0.13	2.5725	0.527
SHMP	1	5.37	1.51	0.57	0.31	0.12	2.5115	0.502
SHMP	1	5.61	2.41	0.48	0.38	0.14	2.5575	0.500
SHMP	1	5.61	2.47	0.50	0.38	0.12	2.4870	0.000
SHMP	1	5.61	2.39	0.53	0.39	0.08	2.7705	0.500
SHMP	1	5.61	2.42	0.53	0.40	0.07	2.5490	0.000
SHMP	50	4.94	2.63	0.52	0.36	0.12	0.4515	10.580
SHMP	50	4.94	2.56	0.46	0.37	0.17	0.3495	11.230
SHMP	50	4.94	2.34	0.49	0.41	0.10	1.5685	10.500
SHMP	50	4.94	2.67	0.49	0.39	0.11	0.5780	7.258
SHMP	50	5.13	2.95	0.51	0.39	0.10	0.2450	15.690
SHMP	50	5.13	2.54	0.50	0.40	0.11	0.2850	15.380
SHMP	50	5.13	2.94	0.48	0.40	0.12	0.2225	14.940
SHMP	50	5.13	2.96	0.45	0.40	0.15	0.2640	15.320
SHMP	50	5.37	3.07	0.44	0.39	0.17	0.1955	6.932
SHMP	50	5.37	2.55	0.46	0.41	0.13	1.0050	6.695
SHMP	50	5.37	3.08	0.51	0.37	0.12	0.1980	6.608
SHMP	50	5.37	3.05	0.48	0.37	0.14	0.2215	6.276
SHMP	50	5.61	3.07	0.44	0.40	0.16	0.1565	7.281
SHMP	50	5.61	3.09	0.48	0.37	0.15	0.1575	7.269
SHMP	50	5.61	3.12	0.51	0.37	0.11	0.1730	7.224
SHMP	50	5.61	3.08	0.53	0.36	0.10	0.1635	9.718
SHMP	100	4.94	3.05	0.48	0.35	0.17	0.3250	17.660
SHMP	100	4.94	3.13	0.50	0.39	0.12	0.5075	17.500
SHMP	100	4.94	2.14	0.47	0.45	0.08	0.2985	17.210
SHMP	100	4.94	3.09	0.52	0.36	0.12	0.3910	17.430
SHMP	100	5.13	2.42	0.45	0.43	0.12	0.1815	0.506

Chelator	Level	pH	Protein	$\alpha$ casein	$\beta$	$\kappa$	Turbidity	Particle Size
SHMP	100	5.13	2.79	0.48	0.39	0.13	0.1855	0.497
SHMP	100	5.13	2.72	0.53	0.37	0.10	0.2085	0.517
SHMP	100	5.13	2.83	0.52	0.37	0.11	0.1945	0.506
SHMP	100	5.37	2.97	0.46	0.39	0.15	0.1770	194.000
SHMP	100	5.37	3.14	0.51	0.37	0.12	0.1490	229.800
SHMP	100	5.37	2.84	0.52	0.34	0.13	0.3105	224.600
SHMP	100	5.37	3.06	0.52	0.38	0.10	0.1775	208.100
SHMP	100	5.61	3.12	0.48	0.37	0.16	0.1325	6.581
SHMP	100	5.61	2.85	0.47	0.41	0.12	0.1590	6.518
SHMP	100	5.61	3.10	0.47	0.40	0.13	0.1610	6.267
SHMP	100	5.61	3.03	0.52	0.37	0.11	0.1655	6.451
TSC	1	4.94	0.63				0.6150	0.704
TSC	1	4.94	0.63				0.1085	0.686
TSC	1	4.94	0.64				0.4195	0.710
TSC	1	4.94	0.69				0.3205	0.700
TSC	1	5.13	0.70	0.48	0.38	0.15	1.6900	0.526
TSC	1	5.13	0.75	0.71	0.29	0.00	2.1050	0.539
TSC	1	5.13	0.70				2.7070	0.537
TSC	1	5.13	0.71	0.48	0.42	0.10	2.2475	0.538
TSC	1	5.37	2.44	0.51	0.37	0.13	2.5755	0.526
TSC	1	5.37	2.35	0.44	0.43	0.13	2.4675	0.861
TSC	1	5.37	2.42	0.51	0.36	0.13	2.7770	0.527
TSC	1	5.37	2.39	0.54	0.34	0.12	2.7020	0.527
TSC	1	5.61	2.62	0.47	0.40	0.13	2.4815	0.552
TSC	1	5.61	2.87	0.50	0.38	0.12	2.4855	0.551
TSC	1	5.61	2.78	0.44	0.42	0.15	2.7990	0.553
TSC	1	5.61	2.70	0.52	0.36	0.12	2.5955	0.552
TSC	50	4.94	1.22				0.2080	6.396
TSC	50	4.94	0.88	0.51	0.49	0.00	1.1320	0.568
TSC	50	4.94	1.19				0.5200	0.620
TSC	50	4.94	1.02				0.6865	0.591
TSC	50	5.13	2.12	0.48	0.41	0.11	2.6390	0.559
TSC	50	5.13	1.94	0.48	0.40	0.12	2.7200	0.576
TSC	50	5.13	2.08	0.48	0.44	0.08	2.6595	0.597
TSC	50	5.13	1.90	0.55	0.39	0.06	2.7780	0.577
TSC	50	5.37	2.73	0.47	0.39	0.15	2.5315	0.596
TSC	50	5.37	2.43	0.48	0.33	0.18	1.5640	0.588
TSC	50	5.37	2.58	0.49	0.41	0.10	2.4170	0.605
TSC	50	5.37	2.56	0.50	0.37	0.13	2.5045	0.595
TSC	50	5.61	2.47	0.46	0.39	0.15	0.8020	0.576

Chelator	Level	pH	Protein	$\alpha$ casein	$\beta$	$\kappa$	Turbidity	Particle Size
TSC	50	5.61	2.09	0.47	0.34	0.19	0.4465	0.565
TSC	50	5.61	2.17	0.47	0.39	0.15	1.4105	0.575
TSC	50	5.61	2.23	0.51	0.40	0.09	0.9515	0.572
TSC	100	4.94	2.07	0.48	0.41	0.12	2.2770	0.728
TSC	100	4.94	1.87	0.49	0.35	0.16	1.6480	0.682
TSC	100	4.94	2.07				2.3600	0.751
TSC	100	4.94	1.98				2.2985	0.716
TSC	100	5.13	2.17	0.49	0.36	0.15	2.2590	0.570
TSC	100	5.13	2.47	0.52	0.35	0.13	1.6925	0.000
TSC	100	5.13	2.10	0.51	0.41	0.08	2.0080	0.637
TSC	100	5.13	2.11	0.55	0.34	0.12	1.9865	0.735
TSC	100	5.37	2.90	0.52	0.34	0.14	1.0960	0.452
TSC	100	5.37	2.72	0.48	0.37	0.14	0.4965	0.552
TSC	100	5.37	2.84	0.49	0.37	0.14	0.5850	0.542
TSC	100	5.37	2.90	0.49	0.34	0.17	0.4990	0.475
TSC	100	5.61	2.89	0.52	0.34	0.13	0.3520	0.545
TSC	100	5.61	2.72	0.49	0.41	0.11	0.2170	0.550
TSC	100	5.61	2.90	0.47	0.38	0.16	0.3155	0.530
TSC	100	5.61	2.77	0.52	0.38	0.11	0.2780	0.541
Control		4.94	0.61				1.5225	5.494
Control		4.94	0.74				0.4665	5.395
Control		4.94	0.70				1.4210	5.671
Control		4.94	0.69				1.5620	5.517
Control		5.13	2.78	0.47	0.40	0.13	0.7380	6.513
Control		5.13	0.80	0.49	0.47	0.04	0.2825	6.495
Control		5.13	0.86				2.7325	6.510
Control		5.13	0.81				2.6820	6.520
Control		5.37	2.29	0.48	0.41	0.11	2.5925	6.371
Control		5.37	0.91	0.45	0.50	0.05	2.6585	6.439
Control		5.37	2.55	0.52	0.41	0.07	2.7480	6.366
Control		5.37	2.55	0.48	0.43	0.09	2.7110	6.293
Control		5.61	2.53	0.53	0.35	0.13	2.6770	0.491
Control		5.61	2.14	0.51	0.37	0.13	2.7825	0.000
Control		5.61	2.57	0.53	0.36	0.12	2.8240	0.503
Control		5.61	2.38	0.46	0.42	0.12	2.7820	0.482

## Appendix E- Unseparated Sample Minitab Output

### 1. Statistical data for particle size analysis



### One-way ANOVA: Particle Size versus Chelator

Equal variances were assumed for the analysis.

#### Factor Information

Factor	Levels	Values
Chelator	7	Control, SHMP 1, SHMP 2, SHMP 3, TSC 1, TSC 2, TSC 3

#### Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Chelator	6	443.20	73.867	41.96	0.000
Error	21	36.97	1.760		
Total	27	480.17			

#### Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.32679	92.30%	90.10%	86.31%

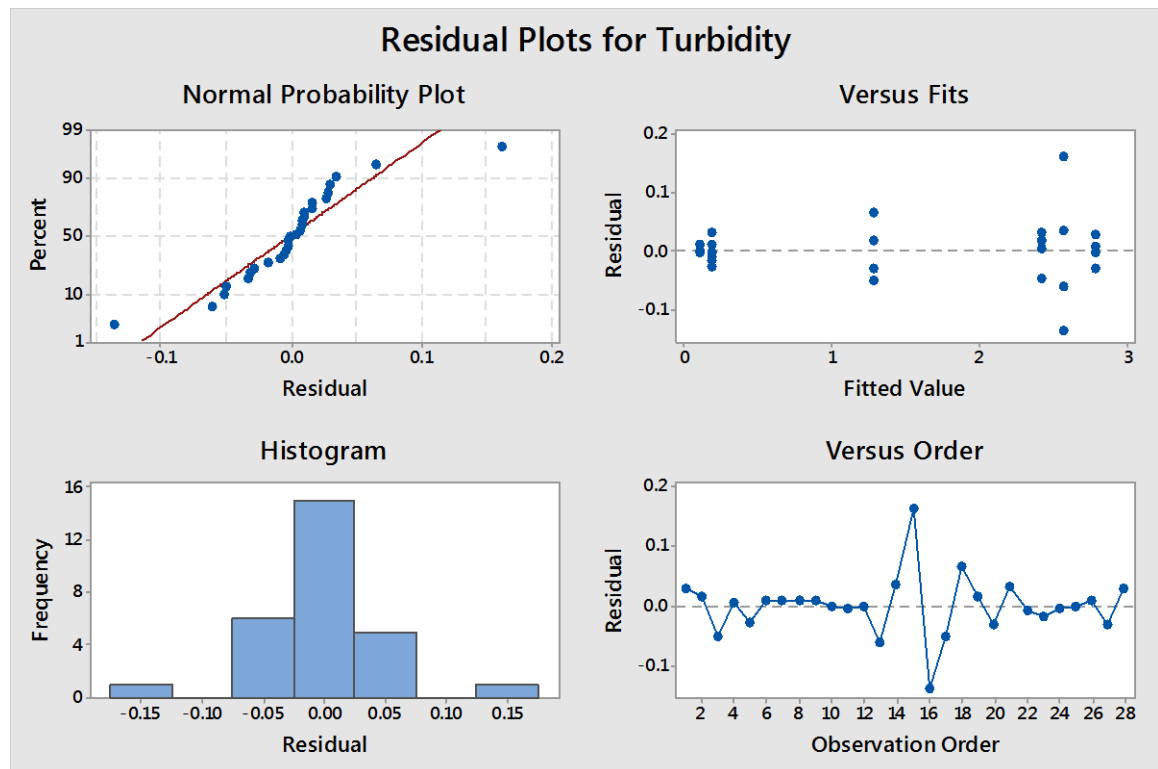
### Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Chelator	N	Mean	Grouping
SHMP 3	4	11.86	A
SHMP 2	4	0.6577	B
TSC 3	4	0.5800	B
TSC 1	4	0.43975	B
TSC 2	4	0.43175	B
SHMP 1	4	0.42275	B
Control	4	0.41625	B

Means that do not share a letter are significantly different.

## 2. Statistical data for turbidity



### One-way ANOVA: Turbidity versus Chelator

Equal variances were assumed for the analysis.

#### Factor Information

Factor	Levels	Values
Chelator	7	Control, SHMP 1, SHMP 2, SHMP 3, TSC 1, TSC 2, TSC 3

#### Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Chelator	6	36.0659	6.01098	1905.79	0.000
Error	21	0.0662	0.00315		
Total	27	36.1321			

#### Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0561610	99.82%	99.76%	99.67%

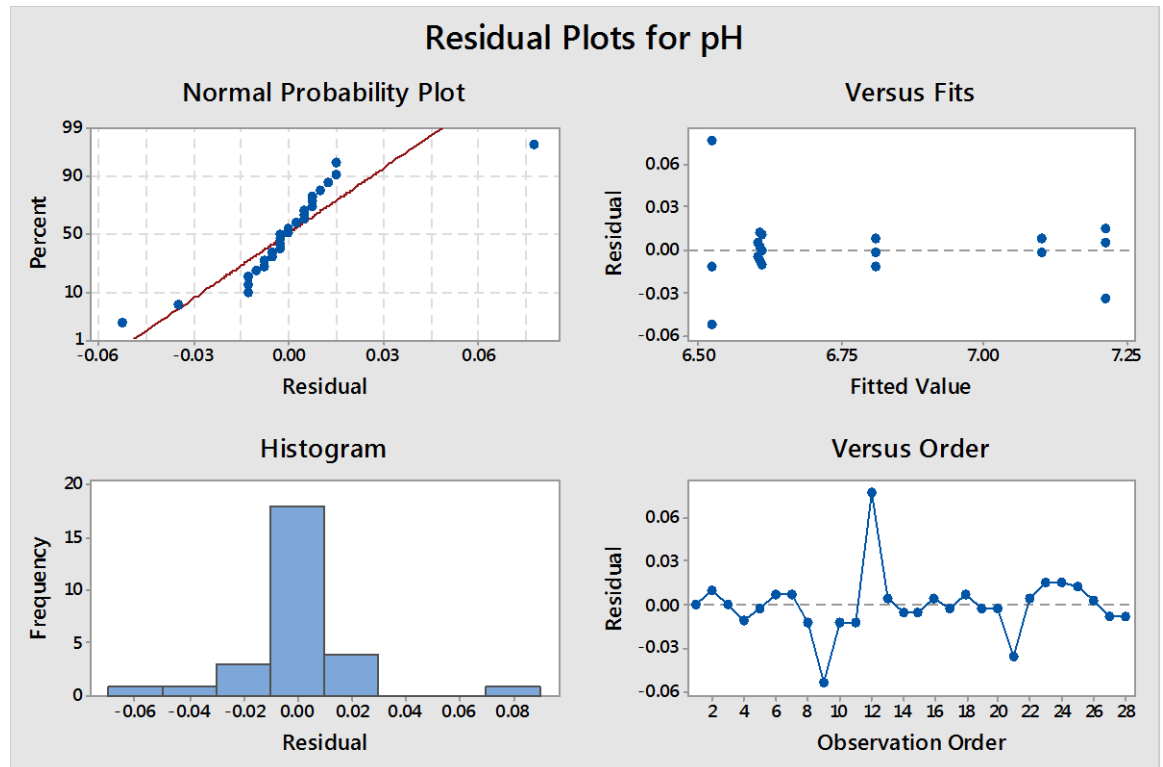
### Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Chelator	N	Mean	Grouping
Control	4	2.7925	A
TSC 1	4	2.5808	B
SHMP 1	4	2.4218	C
TSC 2	4	1.2820	D
TSC 3	4	0.1920	E
SHMP 2	4	0.17900	E
SHMP 3	4	0.10225	E

Means that do not share a letter are significantly different.

### 3. Statistical data for pH



### One-way ANOVA: pH versus Chelator

Equal variances were assumed for the analysis.

#### Factor Information

Factor	Levels	Values
Chelator	7	Control, SHMP 1, SHMP 2, SHMP 3, TSC 1, TSC 2, TSC 3

#### Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Chelator	6	1.79937	0.299895	538.27	0.000
Error	21	0.01170	0.000557		
Total	27	1.81107			

#### Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0236039	99.35%	99.17%	98.85%

### Tukey Pairwise Comparisons



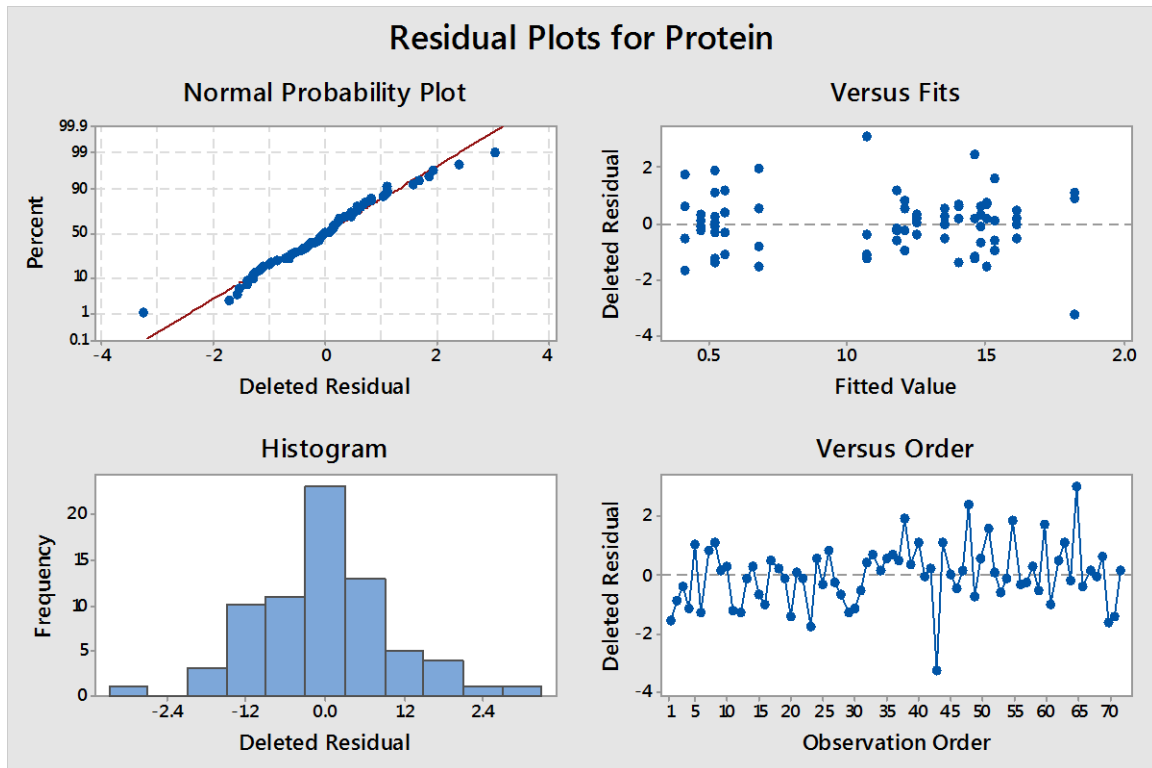
Grouping Information Using the Tukey Method and 95% Confidence

Chelator	N	Mean	Grouping
TSC 3	4	7.2150	A
TSC 2	4	7.10250	B
SHMP 2	4	6.81250	C
SHMP 1	4	6.61000	D
Control	4	6.60750	D
TSC 1	4	6.60500	D
SHMP 3	4	6.5225	E

Means that do not share a letter are significantly different.

## Appendix F- Centrifugation Study Minitab Outputs

### 1. Statistical data for protein content



### General Linear Model: Protein versus Chelator, Level, Force

#### Factor Information

Factor	Type	Levels	Values
Chelator	Fixed	2	SHMP, TSC
Level	Fixed	3	1, 50, 100
Force	Fixed	3	30000, 60000, 90000

#### Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Chelator	1	0.3602	0.36017	172.83	0.000
Level	2	12.6814	6.34068	3042.65	0.000
Force	2	0.4455	0.22274	106.88	0.000
Chelator*Level	2	0.5001	0.25006	120.00	0.000
Chelator*Force	2	0.0708	0.03542	17.00	0.000
Level*Force	4	0.1842	0.04605	22.10	0.000
Chelator*Level*Force	4	0.2575	0.06437	30.89	0.000
Error	54	0.1125	0.00208		
Total	71	14.6122			

# Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0456502	99.23%	98.99%	98.63%

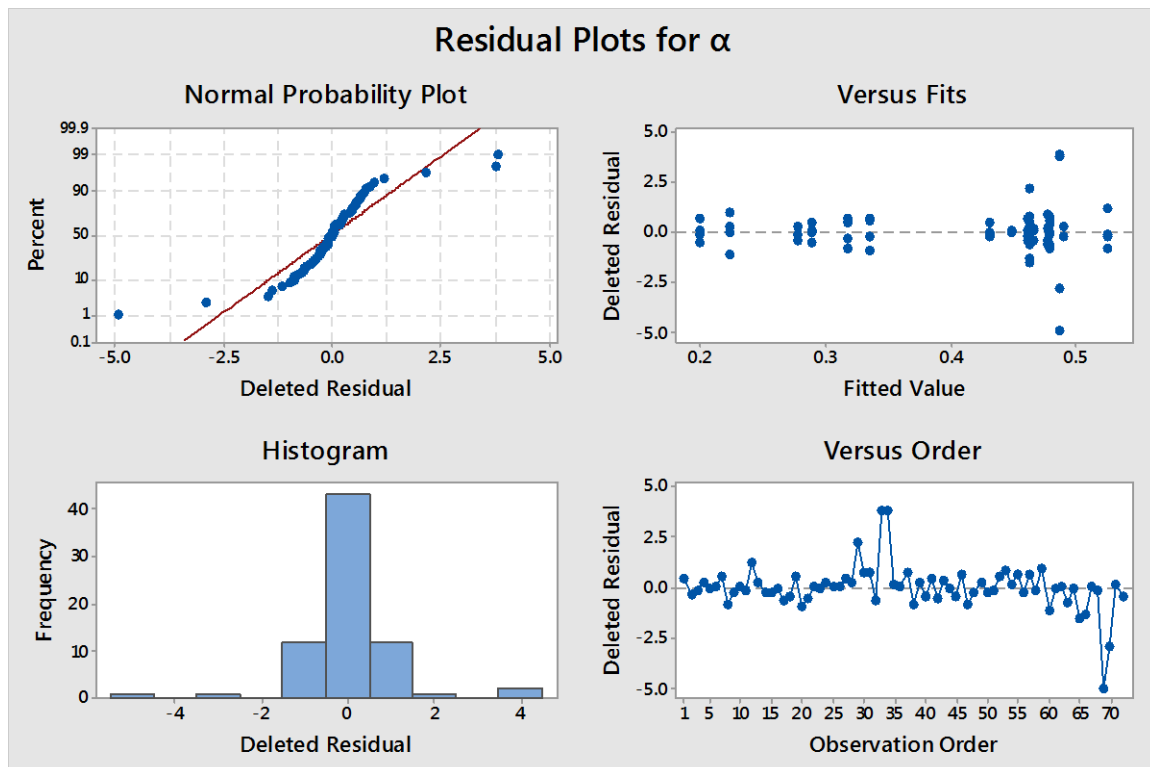
## Tukey Pairwise Comparisons: Response = Protein, Term = Chelator\*Level\*Force

Grouping Information Using the Tukey Method and 95% Confidence

Chelator*Level*Force	N	Mean	Grouping
SHMP 50 30000	4	1.82737	A
TSC 100 30000	4	1.61386	B
SHMP 100 60000	4	1.53846	B C
TSC 100 60000	4	1.50853	B C D
SHMP 100 30000	4	1.48690	C D
SHMP 50 90000	4	1.46061	C D E
TSC 100 90000	4	1.40803	D E
SHMP 100 90000	4	1.35478	E F
SHMP 50 60000	4	1.25104	F G
TSC 50 30000	4	1.21427	G
TSC 50 60000	4	1.18164	G H
TSC 50 90000	4	1.07444	H
SHMP 1 30000	4	0.68090	I
SHMP 1 60000	4	0.55818	J
SHMP 1 90000	4	0.52610	J K
TSC 1 30000	4	0.52361	J K
TSC 1 60000	4	0.47362	J K
TSC 1 90000	4	0.41326	K

Means that do not share a letter are significantly different.

## 2. Statistical data for $\alpha$ casein proportion



## General Linear Model: $\alpha$ versus Chelator, Level, Force

### Factor Information

Factor	Type	Levels	Values
Chelator	Fixed	2	SHMP, TSC
Level	Fixed	3	1, 50, 100
Force	Fixed	3	30000, 60000, 90000

### Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Chelator	1	0.007704	0.007704	5.31	0.025
Level	2	0.636476	0.318238	219.49	0.000
Force	2	0.017588	0.008794	6.07	0.004
Chelator*Level	2	0.009109	0.004555	3.14	0.051
Chelator*Force	2	0.005840	0.002920	2.01	0.143
Level*Force	4	0.024300	0.006075	4.19	0.005
Chelator*Level*Force	4	0.014907	0.003727	2.57	0.048
Error	54	0.078294	0.001450		
Total	71	0.794218			

### Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0380773	90.14%	87.04%	82.47%

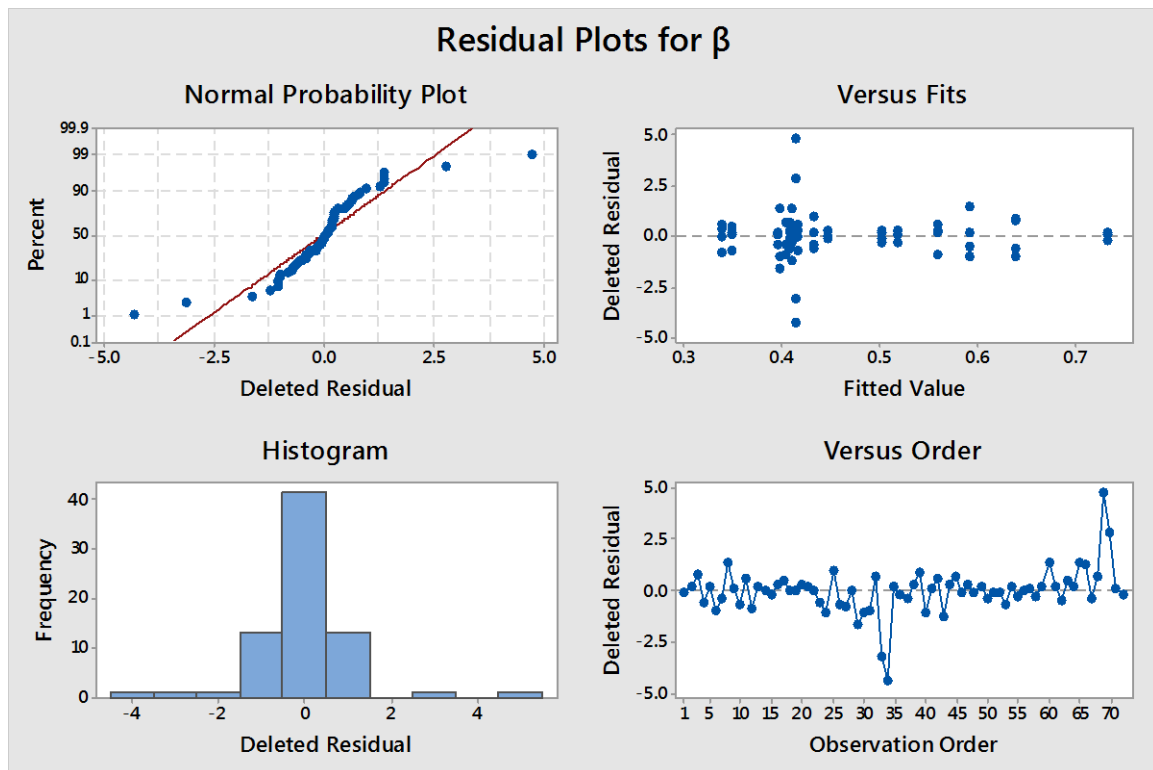
## Tukey Pairwise Comparisons: Response = $\alpha$ , Term = Level\*Force

Grouping Information Using the Tukey Method and 95% Confidence

Level*Force	N	Mean	Grouping
50 90000	8	0.494594	A
100 30000	8	0.485070	A
100 90000	8	0.472309	A
50 30000	8	0.464986	A
50 60000	8	0.462661	A
100 60000	8	0.459943	A
1 30000	8	0.326367	B
1 90000	8	0.256028	C
1 60000	8	0.239055	C

Means that do not share a letter are significantly different.

### 3. Statistical data for $\beta$ casein proportion



### General Linear Model: $\beta$ versus Chelator, Level, Force

#### Factor Information

Factor	Type	Levels	Values
Chelator	Fixed	2	SHMP, TSC
Level	Fixed	3	1, 50, 100
Force	Fixed	3	30000, 60000, 90000

#### Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Chelator	1	0.015600	0.015600	8.49	0.005
Level	2	0.571396	0.285698	155.42	0.000
Force	2	0.073212	0.036606	19.91	0.000
Chelator*Level	2	0.004020	0.002010	1.09	0.342
Chelator*Force	2	0.003840	0.001920	1.04	0.359
Level*Force	4	0.078551	0.019638	10.68	0.000
Chelator*Level*Force	4	0.014697	0.003674	2.00	0.108
Error	54	0.099263	0.001838		
Total	71	0.860578			

#### Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0428743	88.47%	84.83%	79.49%

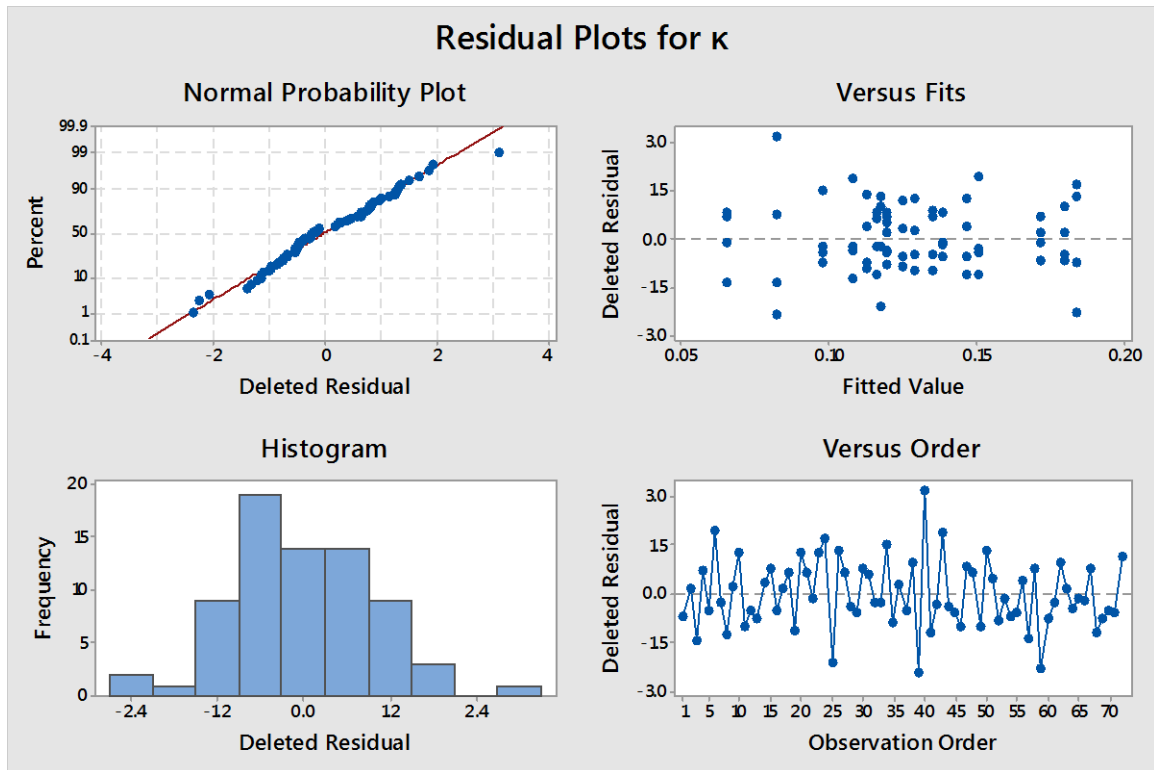
## Tukey Pairwise Comparisons: Response = $\beta$ , Term = Level\*Force

Grouping Information Using the Tukey Method and 95% Confidence

Level*Force	N	Mean	Grouping
1 60000	8	0.687233	A
1 90000	8	0.576510	B
1 30000	8	0.510374	B
100 60000	8	0.431372	C
50 30000	8	0.421934	C
50 60000	8	0.412790	C
100 30000	8	0.400449	C
100 90000	8	0.379408	C
50 90000	8	0.368507	C

Means that do not share a letter are significantly different.

#### 4. Statistical data for $\kappa$ casein proportion



#### General Linear Model: $\kappa$ versus Chelator, Level, Force

##### Factor Information

Factor	Type	Levels	Values
Chelator	Fixed	2	SHMP, TSC
Level	Fixed	3	1, 50, 100
Force	Fixed	3	30000, 60000, 90000

##### Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Chelator	1	0.001378	0.001378	3.24	0.077
Level	2	0.001770	0.000885	2.08	0.135
Force	2	0.028520	0.014260	33.52	0.000
Chelator*Level	2	0.001611	0.000805	1.89	0.161
Chelator*Force	2	0.000516	0.000258	0.61	0.549
Level*Force	4	0.025936	0.006484	15.24	0.000
Chelator*Level*Force	4	0.007300	0.001825	4.29	0.004
Error	54	0.022975	0.000425		
Total	71	0.090007			

##### Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0206268	74.47%	66.44%	54.62%



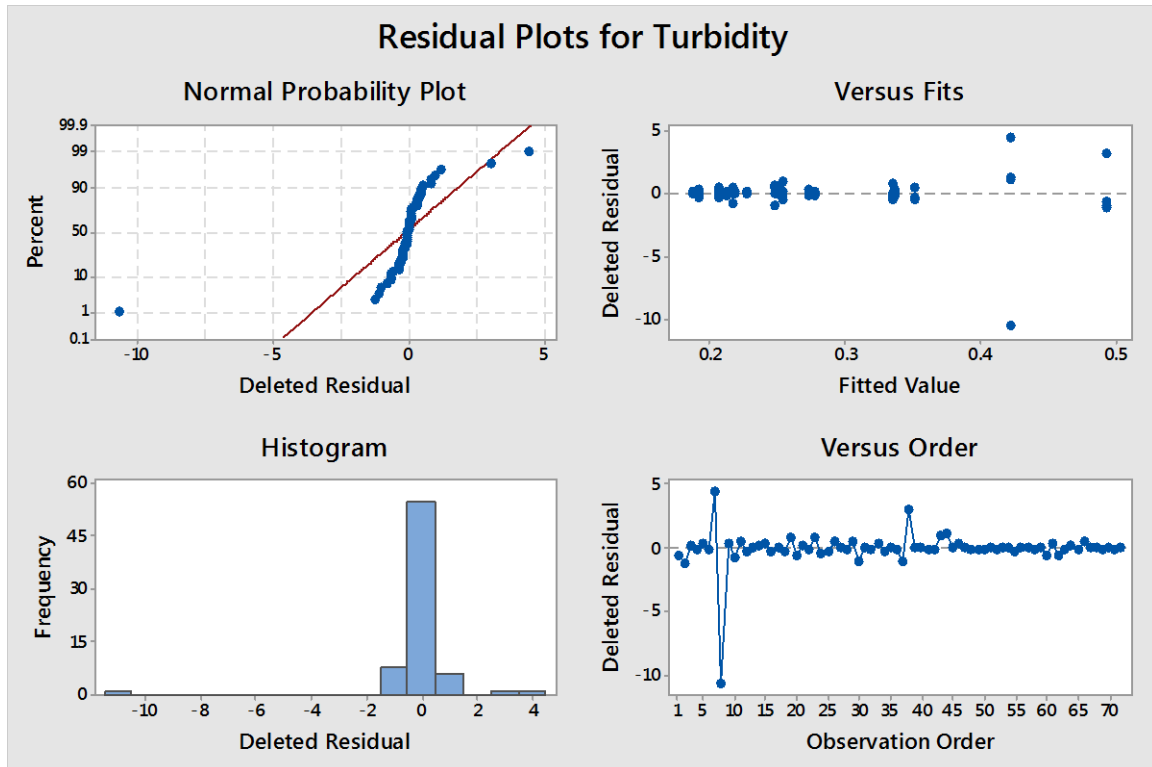
## Tukey Pairwise Comparisons: Response = $\kappa$ , Term = Chelator\*Level\*Force

Grouping Information Using the Tukey Method and 95% Confidence

Chelator*Level*Force	N	Mean	Grouping						
TSC 1 90000	4	0.184259	A						
SHMP 1 30000	4	0.179925	A	B					
SHMP 100 90000	4	0.171742	A	B	C				
SHMP 1 90000	4	0.150665	A	B	C	D			
TSC 1 30000	4	0.146592	A	B	C	D			
TSC 50 90000	4	0.138405	A	B	C	D			
SHMP 50 90000	4	0.135394	A	B	C	D	E		
SHMP 50 60000	4	0.129277		B	C	D	E		
TSC 100 90000	4	0.124826			C	D	E		
TSC 50 60000	4	0.119821			C	D	E		
SHMP 100 60000	4	0.119376			C	D	E		
TSC 50 30000	4	0.117825				D	E	F	
TSC 100 30000	4	0.116302				D	E	F	
SHMP 100 30000	4	0.112661				D	E	F	
SHMP 50 30000	4	0.108335				D	E	F	
TSC 100 60000	4	0.097995				D	E	F	
SHMP 1 60000	4	0.082415					E	F	
TSC 1 60000	4	0.065008							F

Means that do not share a letter are significantly different.

## 5. Statistical data for turbidity



### General Linear Model: Turbidity versus Chelator, Level, Force

#### Factor Information

Factor	Type	Levels	Values
Chelator	Fixed	2	SHMP, TSC
Level	Fixed	3	1, 50, 100
Force	Fixed	3	30000, 60000, 90000

#### Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Chelator	1	0.000018	0.000018	0.01	0.921
Level	2	0.157220	0.078610	42.99	0.000
Force	2	0.148265	0.074132	40.54	0.000
Chelator*Level	2	0.024912	0.012456	6.81	0.002
Chelator*Force	2	0.036081	0.018041	9.87	0.000
Level*Force	4	0.080754	0.020189	11.04	0.000
Chelator*Level*Force	4	0.033111	0.008278	4.53	0.003
Error	54	0.098752	0.001829		
Total	71	0.579114			

#### Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0427639	82.95%	77.58%	69.68%

## Tukey Pairwise Comparisons: Response = Turbidity, Term = Chelator\*Level\*Force

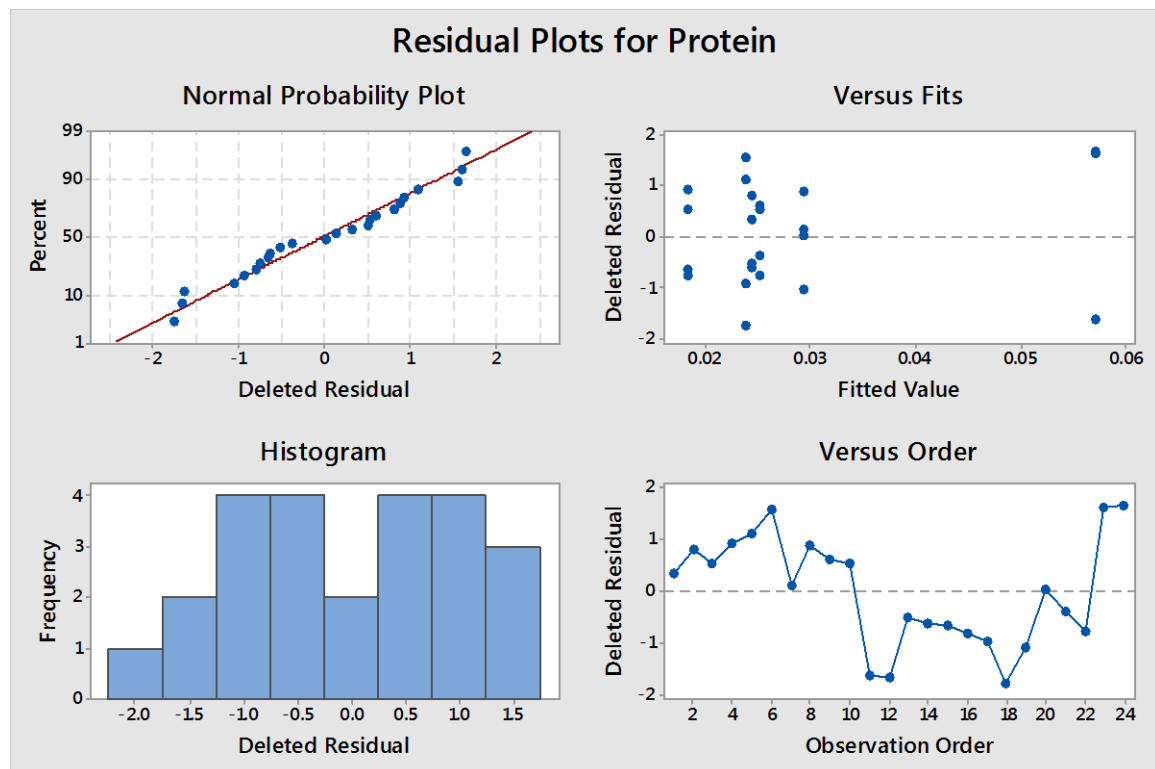
Grouping Information Using the Tukey Method and 95% Confidence

Chelator*Level*Force	N	Mean	Grouping			
SHMP 1 30000	4	0.492125	A			
SHMP 50 30000	4	0.421625	A	B		
TSC 50 30000	4	0.351750		B	C	
TSC 50 60000	4	0.335875		B	C	D
TSC 1 30000	4	0.335625		B	C	D
SHMP 1 60000	4	0.277875			C	D E
TSC 1 60000	4	0.273250			C	D E
TSC 1 90000	4	0.254000			C	D E
SHMP 1 90000	4	0.252750			C	D E
TSC 50 90000	4	0.247625			C	D E
TSC 100 30000	4	0.227250				D E
TSC 100 90000	4	0.218500				E
SHMP 50 60000	4	0.216875				E
SHMP 100 90000	4	0.212000				E
TSC 100 60000	4	0.207000				E
SHMP 50 90000	4	0.206250				E
SHMP 100 60000	4	0.192250				E
SHMP 100 30000	4	0.188125				E

Means that do not share a letter are significantly different.

## Appendix G- Membrane Filtration Study Minitab Outputs

### 1. Statistical data for protein content



### General Linear Model: Protein versus Chelator, Level

#### Factor Information

Factor	Type	Levels	Values
Chelator	Fixed	2	SHMP, TSC
Level	Fixed	3	1, 2, 3

#### Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Chelator	1	0.001361	0.001361	18.68	0.000
Level	2	0.001496	0.000748	10.27	0.001
Chelator*Level	2	0.001003	0.000502	6.88	0.006
Error	18	0.001311	0.000073		
Total	23	0.005172			

#### Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0085358	74.64%	67.60%	54.92%

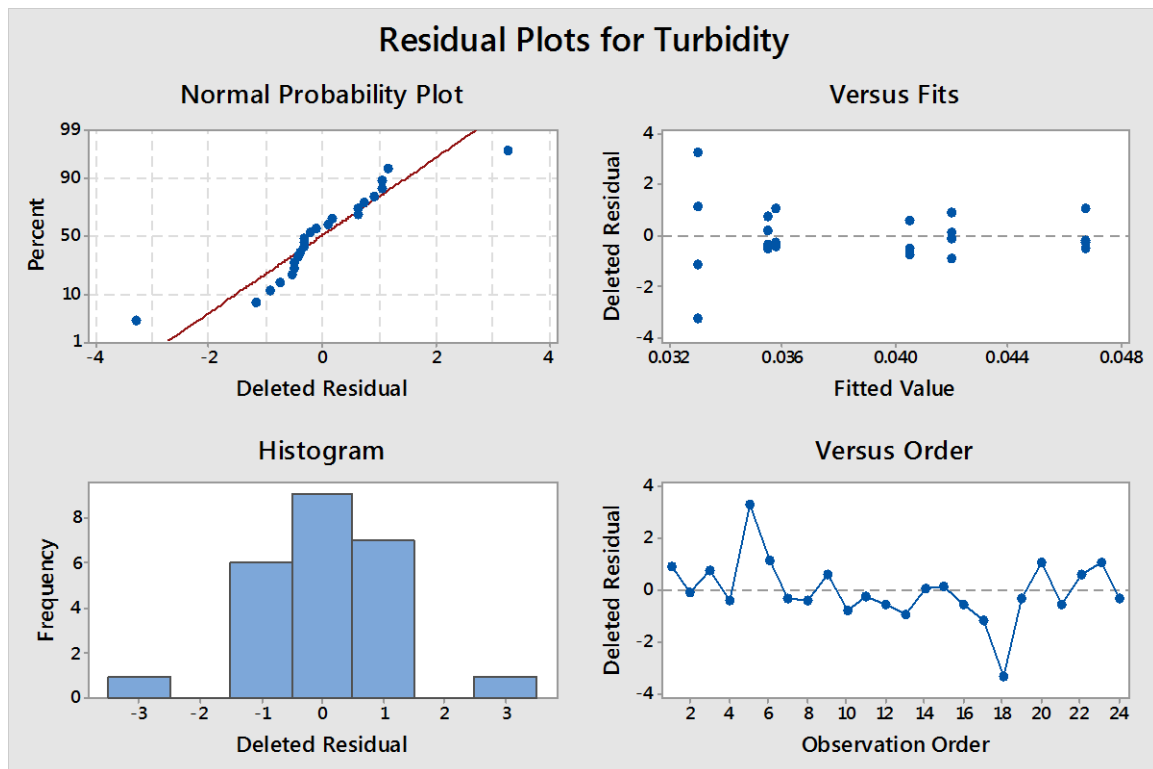
### Tukey Pairwise Comparisons: Response = Protein, Term = Chelator\*Level

Grouping Information Using the Tukey Method and 95% Confidence

Chelator*Level	N	Mean	Grouping
TSC 3	4	0.0572037	A
TSC 1	4	0.0293917	B
TSC 2	4	0.0252977	B
SHMP 1	4	0.0244119	B
SHMP 3	4	0.0238888	B
SHMP 2	4	0.0184084	B

Means that do not share a letter are significantly different.

## 2. Statistical data for turbidity



## General Linear Model: Turbidity versus Chelator, Level

### Factor Information

Factor	Type	Levels	Values
Chelator	Fixed	2	SHMP, TSC
Level	Fixed	3	1, 2, 3

### Analysis of Variance

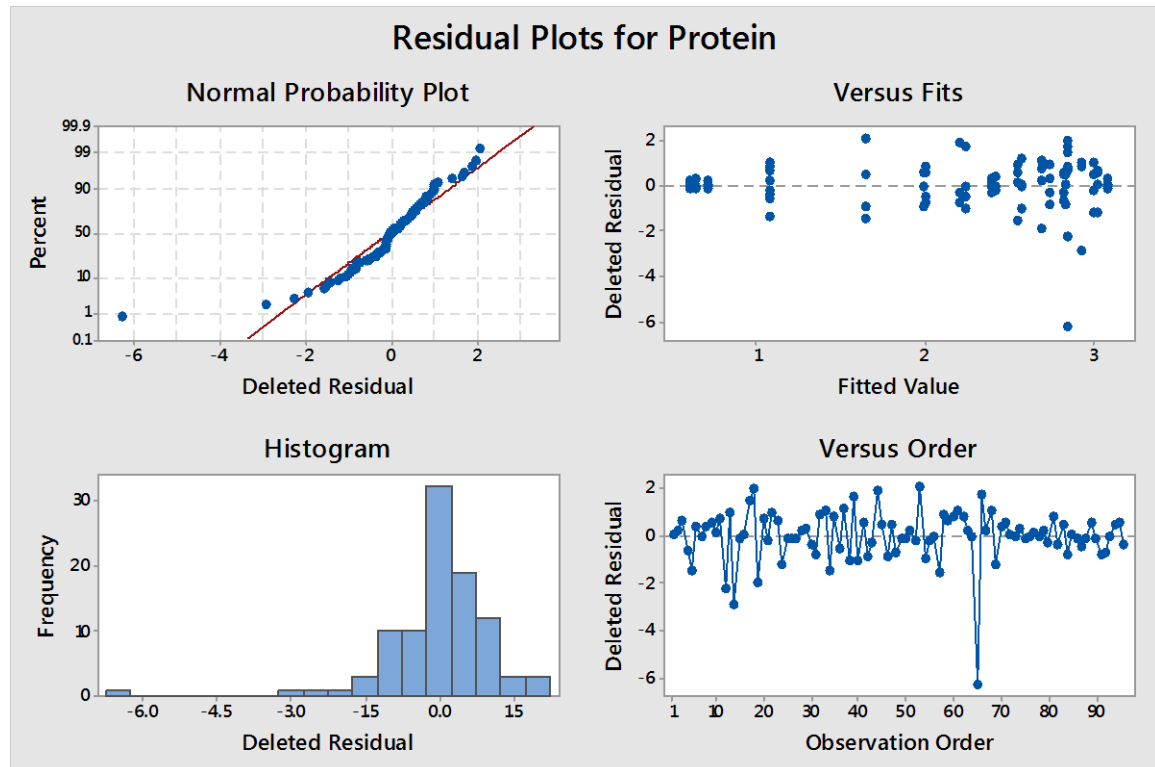
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Chelator	1	0.000104	0.000104	1.03	0.324
Level	2	0.000014	0.000007	0.07	0.933
Chelator*Level	2	0.000402	0.000201	1.99	0.166
Error	18	0.001821	0.000101		
Total	23	0.002342			

### Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0100595	22.22%	0.61%	0.00%

## Appendix H- Acidification Study Minitab Outputs

### 1. Statistical data for protein content



### General Linear Model: Protein versus Chelator, Level, pH (1-low, 4-high)

#### Factor Information

Factor	Type	Levels	Values
Chelator	Fixed	2	SHMP, TSC
Level	Fixed	3	1, 2, 3
pH (1-low, 4-high)	Fixed	4	1, 2, 3, 4

#### Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Chelator	1	3.3391	3.3391	128.20	0.000
Level	2	23.1305	11.5653	444.03	0.000
pH (1-low, 4-high)	3	19.6503	6.5501	251.48	0.000
Chelator*Level	2	4.6057	2.3029	88.41	0.000
Chelator*pH (1-low, 4-high)	3	2.4339	0.8113	31.15	0.000
Level*pH (1-low, 4-high)	6	7.1221	1.1870	45.57	0.000
Chelator*Level*pH (1-low, 4-high)	6	0.7564	0.1261	4.84	0.000
Error	72	1.8753	0.0260		
Total	95	62.9134			

#### Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.161388	97.02%	96.07%	94.70%

## Tukey Pairwise Comparisons: Response = Protein, Term = Chelator\*Level\*pH (1-low, 4-high)

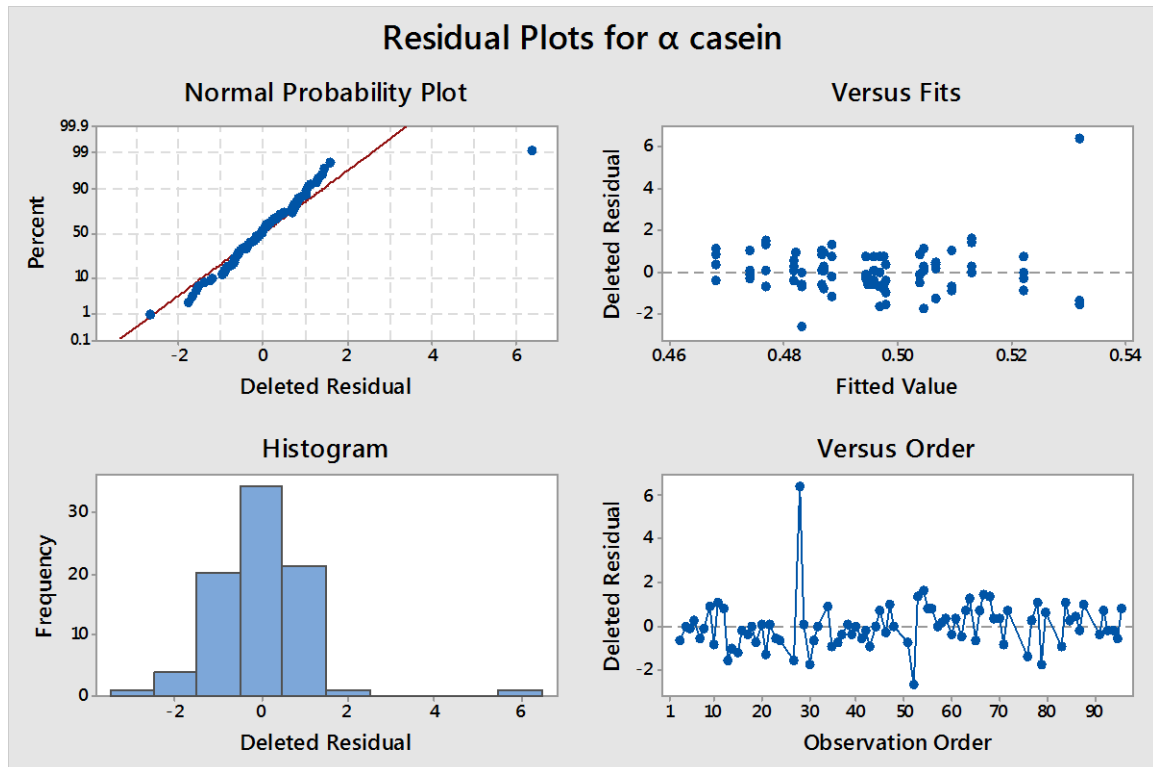
Grouping Information Using the Tukey Method and 95% Confidence

Chelator*Level*pH (1-low, 4-high)	N	Mean	Grouping									
SHMP 2 4	4	3.09068	A									
SHMP 3 4	4	3.02393	A									
SHMP 3 3	4	3.00423	A	B								
SHMP 2 3	4	2.93502	A	B	C							
SHMP 3 1	4	2.85206	A	B	C	D						
SHMP 2 2	4	2.85017	A	B	C	D						
TSC 3 3	4	2.84137	A	B	C	D						
TSC 3 4	4	2.82113	A	B	C	D	E					
TSC 1 4	4	2.74172	A	B	C	D	E					
SHMP 3 2	4	2.69084	A	B	C	D	E					
TSC 2 3	4	2.57642		B	C	D	E	F				
SHMP 2 1	4	2.55207			C	D	E	F				
SHMP 1 4	4	2.42396				D	E	F	G			
TSC 1 3	4	2.39997					E	F	G			
TSC 2 4	4	2.23867						F	G			
TSC 3 2	4	2.21282						F	G			
TSC 2 2	4	2.00991							G	H		
TSC 3 1	4	1.99345							G	H		
SHMP 1 3	4	1.64795								H		
SHMP 1 2	4	1.07800									I	
TSC 2 1	4	1.07774									I	
TSC 1 2	4	0.71535									I	J
TSC 1 1	4	0.64762									I	J
SHMP 1 1	4	0.60325										J

Means that do not share a letter are significantly different.



## 2. Statistical data for $\alpha$ casein content



### General Linear Model: $\alpha$ casein versus Chelator, Level, pH (1-low, 4-high)

#### Factor Information

Factor	Type	Levels	Values
Chelator	Fixed	2	SHMP, TSC
Level	Fixed	3	1, 2, 3
pH (1-low, 4-high)	Fixed	4	1, 2, 3, 4

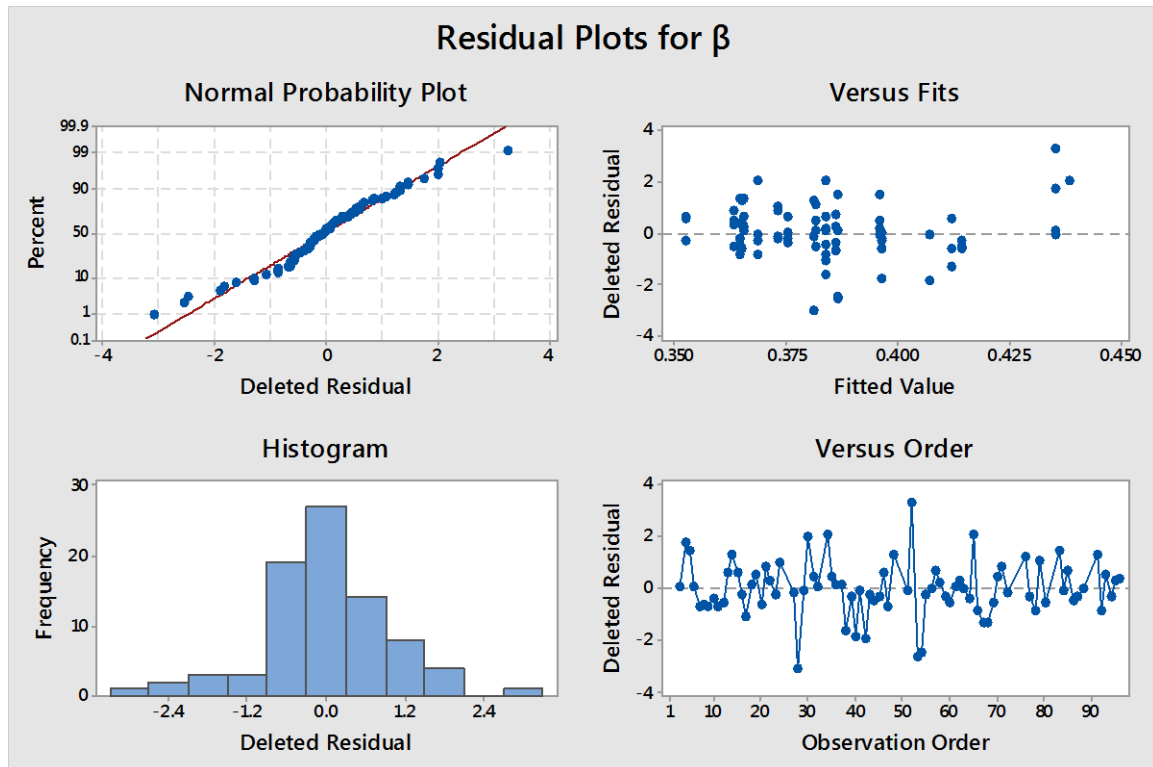
#### Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Chelator	1	0.000423	0.000423	0.28	0.596
Level	2	0.004536	0.002268	1.52	0.226
pH (1-low, 4-high)	3	0.001052	0.000351	0.23	0.872
Chelator*Level	2	0.000174	0.000087	0.06	0.943
Chelator*pH (1-low, 4-high)	3	0.012548	0.004183	2.80	0.046
Error	70	0.104488	0.001493		
Lack-of-Fit	10	0.019485	0.001949	1.38	0.214
Pure Error	60	0.085003	0.001417		
Total	81	0.122998			

#### Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0386354	15.05%	1.70%	0.00%

### 3. Statistical data for $\beta$ casein content



### General Linear Model: $\beta$ versus Chelator, Level, pH (1-low, 4-high)

#### Factor Information

Factor	Type	Levels	Values
Chelator	Fixed	2	SHMP, TSC
Level	Fixed	3	1, 2, 3
pH (1-low, 4-high)	Fixed	4	1, 2, 3, 4

#### Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Chelator	1	0.000172	0.000172	0.14	0.710
Level	2	0.006150	0.003075	2.49	0.090
pH (1-low, 4-high)	3	0.015216	0.005072	4.11	0.010
Chelator*Level	2	0.004851	0.002426	1.97	0.148
Chelator*pH (1-low, 4-high)	3	0.009313	0.003104	2.52	0.065
Error	70	0.086382	0.001234		
Lack-of-Fit	10	0.024674	0.002467	2.40	0.018
Pure Error	60	0.061708	0.001028		
Total	81	0.121128			

#### Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0351288	28.69%	17.48%	0.00%

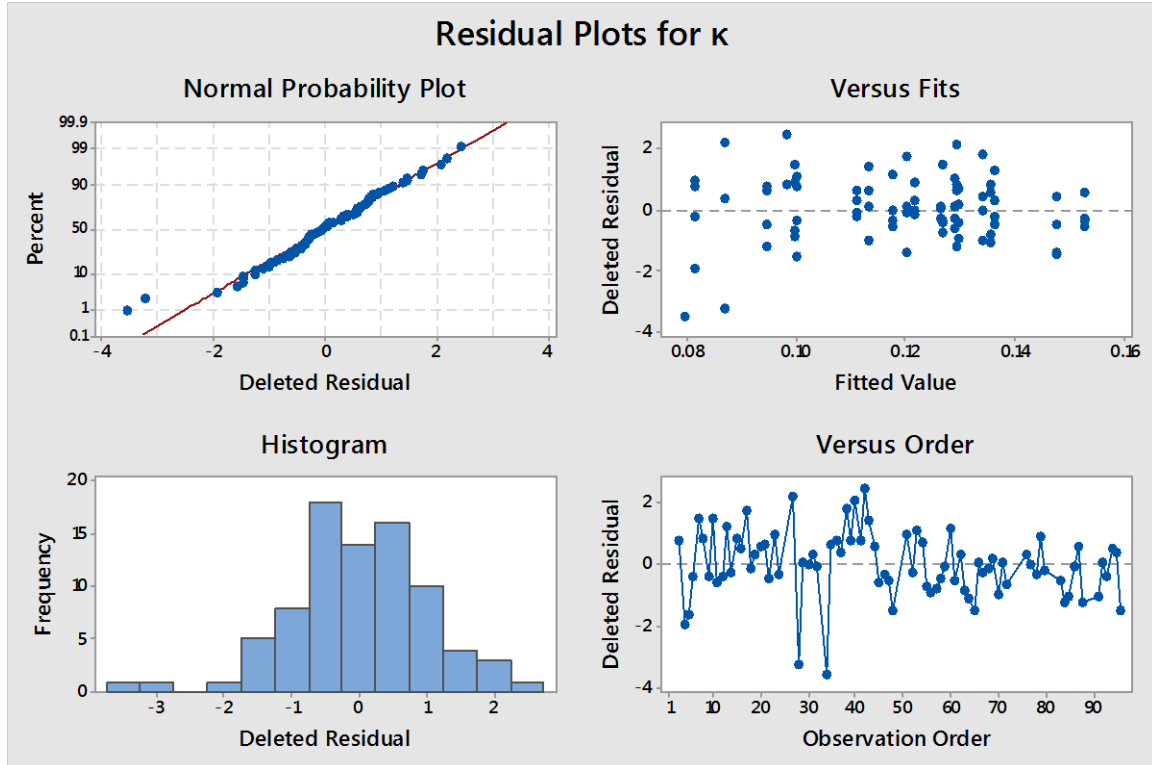
## Tukey Pairwise Comparisons: Response = $\beta$ , Term = pH (1-low, 4-high)

Grouping Information Using the Tukey Method and 95% Confidence

pH (1-low, 4-high)	N	Mean	Grouping
1	11	0.407645	A
2	23	0.400482	A
4	24	0.381295	A B
3	24	0.369996	B

Means that do not share a letter are significantly different.

#### 4. Statistical data for $\kappa$ casein content



#### General Linear Model: $\kappa$ versus Chelator, Level, pH (1-low, 4-high)

##### Factor Information

Factor	Type	Levels	Values
Chelator	Fixed	2	SHMP, TSC
Level	Fixed	3	1, 2, 3
pH (1-low, 4-high)	Fixed	4	1, 2, 3, 4

##### Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Chelator	1	0.000056	0.000056	0.06	0.811
Level	2	0.010254	0.005127	5.30	0.007
pH (1-low, 4-high)	3	0.015635	0.005212	5.38	0.002
Chelator*Level	2	0.003292	0.001646	1.70	0.190
Chelator*pH (1-low, 4-high)	3	0.003921	0.001307	1.35	0.265
Error	70	0.067770	0.000968		
Lack-of-Fit	10	0.013685	0.001369	1.52	0.155
Pure Error	60	0.054085	0.000901		
Total	81	0.097877			

##### Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0311149	30.76%	19.88%	0.00%

#### Tukey Pairwise Comparisons: Response = $\kappa$ , Term = Level

Grouping Information Using the Tukey Method and 95% Confidence

Level	N	Mean	Grouping
3	30	0.125321	A
2	29	0.119341	A
1	23	0.097271	B

Means that do not share a letter are significantly different.

### **Tukey Pairwise Comparisons: Response = $\kappa$ , Term = pH (1-low, 4-high)**

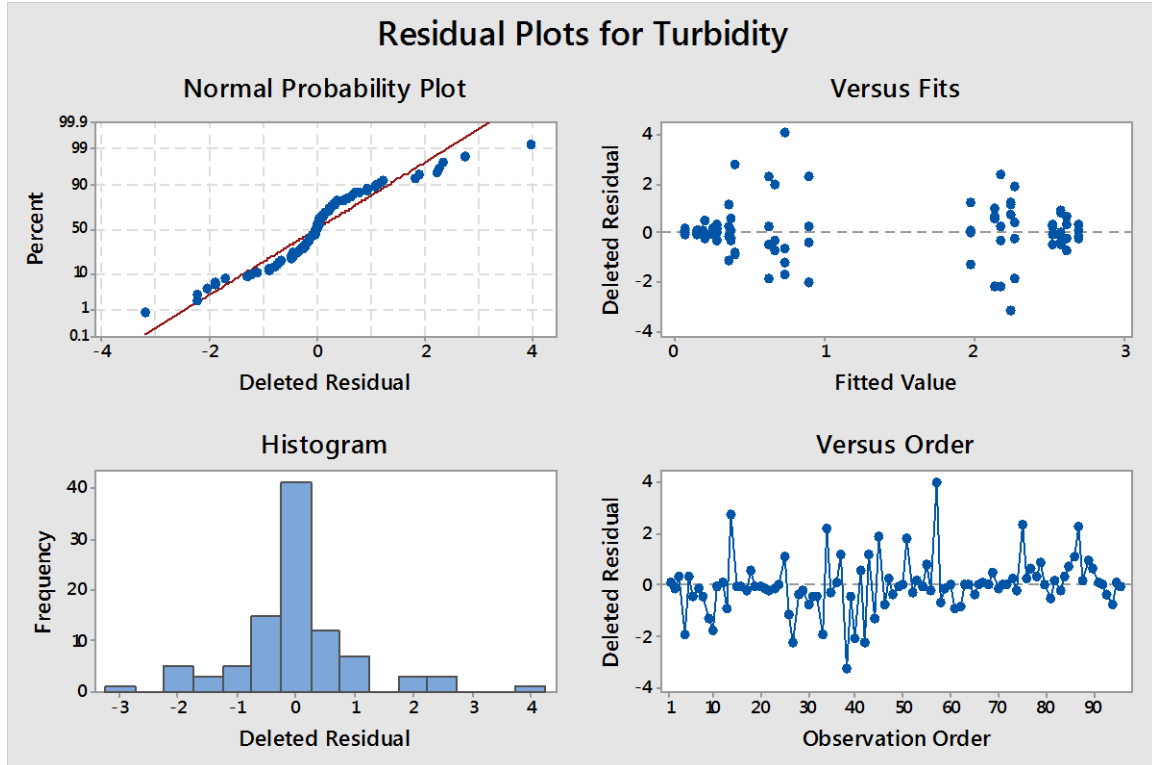
Grouping Information Using the Tukey Method and 95% Confidence

pH (1-low,  
4-high)

	N	Mean	Grouping
3	24	0.130099	A
4	24	0.127304	A
2	23	0.100739	B
1	11	0.097769	A B

Means that do not share a letter are significantly different.

## 5. Statistical data for turbidity



## General Linear Model: Turbidity versus Chelator, Level, pH (1-low, 4-high)

### Factor Information

Factor	Type	Levels	Values
Chelator	Fixed	2	SHMP, TSC
Level	Fixed	3	1, 2, 3
pH (1-low, 4-high)	Fixed	4	1, 2, 3, 4

### Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Chelator	1	14.727	14.7275	211.49	0.000
Level	2	23.474	11.7371	168.55	0.000
pH (1-low, 4-high)	3	10.928	3.6425	52.31	0.000
Chelator*Level	2	6.149	3.0744	44.15	0.000
Chelator*pH (1-low, 4-high)	3	3.695	1.2318	17.69	0.000
Level*pH (1-low, 4-high)	6	31.375	5.2291	75.09	0.000
Chelator*Level*pH (1-low, 4-high)	6	8.697	1.4495	20.82	0.000
Error	72	5.014	0.0696		
Total	95	104.059			

### Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.263886	95.18%	93.64%	91.43%

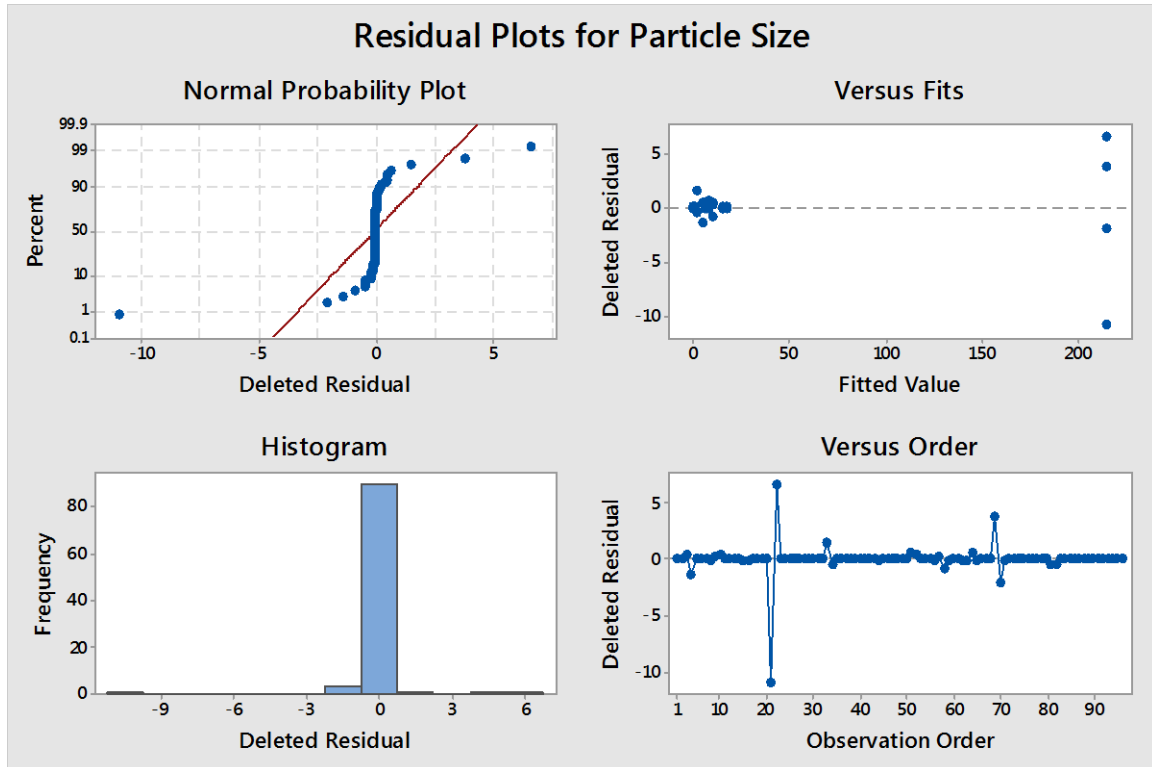
## Tukey Pairwise Comparisons: Response = Turbidity, Term = Chelator\*Level\*pH (1-low, 4-high)

Grouping Information Using the Tukey Method and 95% Confidence

Chelator*Level*pH (1-low, 4-high)	N	Mean	Grouping
TSC 2 2	4	2.69913	A
TSC 1 3	4	2.63050	A B
SHMP 1 4	4	2.59100	A B
TSC 1 4	4	2.59037	A B
SHMP 1 3	4	2.52537	A B
SHMP 1 2	4	2.28375	A B
TSC 2 3	4	2.25425	A B
TSC 1 2	4	2.18737	A B
TSC 3 1	4	2.14587	A B
TSC 3 2	4	1.98650	B
TSC 2 4	4	0.90263	C
SHMP 2 1	4	0.73688	C D
TSC 3 3	4	0.66913	C D
TSC 2 1	4	0.63662	C D
SHMP 2 3	4	0.40500	C D
SHMP 3 1	4	0.38050	C D
TSC 1 1	4	0.36587	C D
TSC 3 4	4	0.29063	C D
SHMP 2 2	4	0.25413	C D
SHMP 3 3	4	0.20350	C D
SHMP 3 2	4	0.19250	D
SHMP 2 4	4	0.16263	D
SHMP 3 4	4	0.15450	D
SHMP 1 1	4	0.06888	D

Means that do not share a letter are significantly different.

## 6. Statistical data for particle size analysis



## General Linear Model: Particle Size versus Chelator, Level, pH (1-low, 4-high)

### Factor Information

Factor	Type	Levels	Values
Chelator	Fixed	2	SHMP, TSC
Level	Fixed	3	1, 2, 3
pH (1-low, 4-high)	Fixed	4	1, 2, 3, 4

### Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Chelator	1	12699	12698.5	1063.33	0.000
Level	2	15674	7837.2	656.26	0.000
pH (1-low, 4-high)	3	20012	6670.7	558.58	0.000
Chelator*Level	2	15870	7934.8	664.43	0.000
Chelator*pH (1-low, 4-high)	3	20191	6730.2	563.57	0.000
Level*pH (1-low, 4-high)	6	44008	7334.7	614.19	0.000
Chelator*Level*pH (1-low, 4-high)	6	43911	7318.4	612.82	0.000
Error	72	860	11.9		
Total	95	173224			

### Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
3.45574	99.50%	99.35%	99.12%



## Tukey Pairwise Comparisons: Response = Particle Size, Term = Chelator\*Level\*pH (1-low, 4-high)

Grouping Information Using the Tukey Method and 95% Confidence

Chelator*Level*pH (1-low, 4-high)	N	Mean	Grouping
SHMP 3 3	4	214.125	A
SHMP 3 1	4	17.450	B
SHMP 2 2	4	15.332	B C
SHMP 2 1	4	9.892	B C D
SHMP 2 4	4	7.873	C D E
SHMP 2 3	4	6.628	C D E
SHMP 3 4	4	6.454	C D E
SHMP 1 2	4	4.697	D E
TSC 2 1	4	2.044	D E
SHMP 1 1	4	0.749	D E
TSC 3 1	4	0.719	D E
TSC 1 1	4	0.700	D E
TSC 1 3	4	0.610	E
TSC 2 3	4	0.596	E
TSC 2 2	4	0.577	E
TSC 2 4	4	0.572	E
TSC 1 4	4	0.552	E
TSC 3 4	4	0.542	E
TSC 1 2	4	0.535	E
SHMP 1 3	4	0.508	E
SHMP 3 2	4	0.507	E
TSC 3 3	4	0.505	E
TSC 3 2	4	0.485	E
SHMP 1 4	4	0.250	E

Means that do not share a letter are significantly different.